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DEPARTMENT OF THE ARMY
TECHNICAL MANUAL

DEPARTMENT OF THE
AIR FORCE MANUAL

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METHODS FOR MEDICAL LABORATORY TECHNICIANS

DEPARTMENTS OF THE ARMY AND THE AIR FORCE

AUGUST 1951

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METHODS FOR MEDICAL LABORATORY TECHNICIANS



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CHAPTER I

BASIC LABORATORY PROCEDURES

Section I. INTRODUCTION

1. Purpose

This manual is intended primarily for use in training medical laboratory technicians in Armed Forces laboratories. It also provides detailed directions for most of the diagnostic clinical laboratory tests required in Armed Forces hospitals or other Armed Forces laboratories. Standardization of technical procedures and the use of standard medical supplies and equipment in Armed Forces laboratories are encouraged.

2. Scope

This manual, originally based on the fourth (1935) edition of *Laboratory Methods of The United States Army*, has been entirely rewritten to meet the different objective. As a training manual for the apprentice technician it has been given detail of technic but abridgment of subject interpretation. Some topics, not ordinarily encountered by the apprentice technician, have been given an entry for general orientation. Owing to limitation of space, most of the less frequently used tests and duplicate or optional tests have been omitted. Where several acceptable tests are available for a single purpose, the one most adaptable to apprentice technicians in Armed Forces laboratories has been described. A number of new approved methods have been added. Free reference has been made to standard texts on the various subjects. (See Appendix.)

Section II. MEDICAL SUPPLIES

3. Standard

These are entered in the current edition of the Armed Services Catalog of Medical Matériel. This list is designed to provide for all ordinary requirements. So far as practicable only standard

items should be used, methods being chosen, when available, which will utilize standard items. Most laboratory supplies are in classes 1 and 4 of this catalog.

a. **EXPENDABLE ITEMS.** These are items such as glass slides which, when they become unserviceable, can be discarded without further accountability. These are indicated by a capital letter "X" in column following the item numbered in the Armed Services Catalog of Medical Matériel.

b. **NONEXPENDABLE ITEMS.** These are items such as tools and instruments, which do not lose their identity by being used. The medical supply officer maintains property records and these items are issued to the laboratory on memorandum receipt, resulting in the medical supply officer being the accountable officer and a laboratory officer the responsible officer. Items of this nature will remain charged to the responsible officer until they are returned to the medical supply for exchange or credit, or disposed of by report of survey.

4. Nonstandard

These items do not appear in the Armed Services Catalog of Medical Matériel either because they are replaceable by some standard item or because their use is so limited as not to warrant storage at supply depots. A few items listed in this manual are nonstandard for the latter reason. When nonstandard items are essential, a request should be made to the medical supply officer, giving full details as to why the item is needed. The medical supply officer in turn will initiate requisition or purchase in compliance with current instructions.

5. Laboratory Supplies

Requisitions are submitted periodically by the medical supply officer of the unit, based upon stock levels as established by the station.

6. Medical Supply Officer

The Chief of Laboratory Service should maintain close liaison with the medical supply officer with reference to laboratory supplies. He should assist the medical supply officer in determining the levels of each item required for the laboratory, basing the requirements on past issue experience and anticipated needs. The medical supply officer will have available for reference all current supply publications.

Section III. LABORATORY REPORTS

7. Purpose

Reports of laboratory examinations are made to give useful information for diagnosis, for guidance to treatment, or for epidemiologic or sanitary guidance. Those reports that pertain to a hospital patient are filed with and become a part of the clinical record of the patient. Those reports having epidemiologic or sanitary reference become a part of the office file of the administrative offices. Duplicate reports become the laboratory office file for future reference. It may be stated as an axiom that a laboratory report, as seen on a patient's record chart, reflects the acumen, industry, and neatness of the laboratory examiner; a neat, concise, and understandable report reflects careful technic in the examination; an untidy, incomplete, or vague report reflects careless or incomplete technical handling. A technician should not impair the value of an examination on which he has spent hours of careful work and thought by terminating it in a few minutes with a hasty, careless report.

8. Preparation

Reports are prepared by the laboratory in duplicate; the original report is forwarded to the ward, office, or officer requesting the examination; the duplicate copy is retained in the laboratory for file. Some reports, such as autopsy protocols and the reports of milk and water analysis, call for multiple copies on special forms.

a. The substance of the report is entered by the technician or officer making the examination and should be exact, understandable, neat, and prompt, giving complete information concerning source, date, special circumstances, result of examination, and date of report.

b. Verification of the report is made by the initials of the examining technician or officer and the signature of the laboratory officer.

c. Reports of laboratory work are strictly confidential; information concerning results of tests is to be released only through medical channels. Caution should be observed in giving out information by telephone.

d. Work notes are kept for all procedures other than those subject to immediate report. These contain a record of the day-by-day observations and procedures and serve as the basis for the final report of the examination. At no time should memory be substituted for a written record of past findings and events.

e. Monthly and annual reports are prepared by the laboratory officer, giving the kinds and numbers of examinations made during the period. These are prepared from the duplicate report forms and from the records of each component part of the laboratory on unreported as well as reported undertakings.

9. Forms

Forms are provided and should be used in reporting results of laboratory work.

Section IV. THE COMPOUND MICROSCOPE

10. Structure

A microscope, the working tool of a microbiologist, consists of four groups of parts, each group composed of a number of units:

a. FRAMEWORK.

- (1) *Base*, on which the microscope rests.
- (2) *Handle*, by which it is carried and which supports the magnifying and adjusted systems.
- (3) *Stage*, a perforated shelf on which the object rests.
- (4) *Mechanical stage*, which moves the object about on the stage.

b. ILLUMINATION SYSTEM.

- (1) *Mirror*, which reflects light upward.
- (2) *Condenser*, placed just beneath hole in stage.
- (3) *Diaphragm*, just beneath condenser, by which the intensity of the light may be adjusted.

c. MAGNIFICATION SYSTEM.

- (1) *Nosepiece*, generally triple, to receive the objectives.
- (2) *Objectives*, generally three, the main magnifying part, designated according to the equivalent focal length as 16, 4 and 1.9 mm. The initial magnifications by these objectives are 10x, 43x, and 95x; the 1.9mm is used in most bacteriologic studies.
- (3) *Body tube and drawtube*, through which the light passes to the ocular.
- (4) *Ocular*, an additional magnifying piece, of which two are generally furnished, a 5x and a 10x. The number indicates the magnification by the ocular of the image formed by the objective at a tube length of 160 mm.
- (5) The *magnification* of any combination of objectives and oculars may be obtained by multiplying the magnification

of the objective by that of the ocular. The magnification given by different combinations of objectives and oculars is as follows:

	5x	10x
16 mm (10x) -----	50	100
4 mm (43x) -----	215	430
1.9 mm (95x) -----	475	950

d. ADJUSTMENT SYSTEM.

- (1) The coarse adjuster gives rapid movement over a wide range and is used to obtain an approximate focus.
- (2) The fine adjuster gives a slow movement over a limited range and is used to obtain an exact focus, after prior coarse adjustment.

II. Use

a. ADJUSTMENT OF LIGHT. A suitable source of light is placed in front of the microscope. It may be daylight (not direct sunlight) or a bright artificial light, and must be intense for the highest magnification. The mirror is adjusted to direct the light upward through the condenser, after which the intensity is adjusted by means of the diaphragm.

b. ADJUSTMENT OF OBJECT. The material to be examined is placed on a glass slide. This is set on the stage, held in the grip of the mechanical stage, and moved around by the latter until the desired areas lie beneath the objective. When the oil immersion lens is used, a small drop of immersion oil is placed on the cover glass or stained smear.

c. ADJUSTMENT OF MAGNIFICATION SYSTEM. The desired objective is rotated into place at the lower end of the body tube. The desired ocular is placed in the upper end of the drawtube. The observer then closely applies an eye to the ocular.

d. ADJUSTMENT OF FOCUS. The number on the objective indicates its "equivalent focal length" in millimeters. If the 16-mm objective were a simple converging lens, the equivalent focal length would also be its working distance (that is, the distance above the object when the lens is in focus) but, for a compound objective, the working distance is always much less than the equivalent focal length. For examples it will be found that the working distance of the 16-mm objective is about 5mm, that of the 4-mm objective less than 1 mm, and that of the 1.9-mm objective less than 0.2 mm. Most microscopes are equipped with parfocal objectives so that if the 16-mm objective is in focus the others will be approximately in focus when the nosepiece is revolved. To focus the 4-mm or 1.9-mm objective, carefully lower

the objective with the coarse adjustment, watching it from the side with the eye on the level of the microscope stage, until the objective nearly touches the coverslip. Then, looking through the ocular, slowly raise the objective until the object can be seen, and bring into sharp focus with the fine adjustment. If necessary, readjust the intensity of the light to give maximal visibility. Never lower the objective with the fine adjustment. The contact cannot be felt if it strikes the slide; if this occurs the slide may be broken, and the lens scratched and ruined.

12. Care

a. Objective and ocular surfaces may be cleaned by a little breath moisture followed by a stroke of lens paper, as in cleaning the lenses of spectacles.

b. Immersion oil, cedarwood oil, or mineral oil (liquid petrolatum), used with the oil-immersion objective, is to be wiped off with soft lens paper after each use, avoiding gauze or other scratching agents.

Caution: Different oils must not be allowed to mix in the oil bottle, on the lens, or on the slide.

c. Cleaning of entire microscope is to be done frequently to remove dust, finger marks, oil, grease, and remnants of specimens.

d. The microscope is to be covered at all times when not in use.

e. Dried oil may be removed by wiping with lens paper soaked with xylene, wiping away surplus at once with dry lens paper. Caution must be used in applying any solvent fluid to the objective; alcohol should never be used.

f. The housing of binocular tubes should never be removed except by an expert, for slight maladjustment of the contained prisms will distort the images.

g. Light machine oil occasionally is applied to the moving parts.

Section V. MANIPULATION OF GLASSWARE

13. Glass Handling

a. **PURPOSE.** Cutting, mending, and simple fusion of glass tubing, preparation of capillary pipettes and vaccine vials, and other simple glass-handling procedures are part of the technical routine of every laboratory. Repair and manufacture of larger and more complicated glassware are beyond the capacity of the average laboratory, requiring expert glass blowers with special equipment.

b. EQUIPMENT. Soft glass can be handled with the ordinary laboratory equipment, using a blast furnace with illuminating gas and foot-power or motor-driven air pressure. Some work can be done with the ordinary Bunsen burner, with or without a fish-tail tip. Glass rods, test tubes, and thick-walled glass tubing of soft glass 6, 8, and 10 mm in diameter are easily cut, bent, and fused. A small triangular file or a glass-knife is used for cutting. The hard glasses, such as Pyrex, have high softening points, and seals cannot be made without special high-temperature blast lamps, although bends in small tubing can be made with the Bunsen burner.

14. Glass Cutting

a. GLASS TUBING AND RODS. Hold the piece of glass firmly on the top of a laboratory table and scratch it sharply in one spot with the edge of a triangular file or hard-steel knife. Then hold the tubing or rod in both hands with the thumbs opposite the nick; exert a slight pull and break with a quick snap; a clean-cut, even break should result. The scratch should be made by passing the file or knife once across the glass, never by "sawing" it back and forth. If one end of the tubing is too short to handle, the break can be made by laying the tube over the upturned edge of a file, with the scratch uppermost and above the file. The short end of the tube is broken off by hitting it sharply just beyond the scratch with another file or a small hammer. Another method is to touch one end of the scratch with a red-hot bead of molten glass; this will usually start a crack that passes around the tube. Finally, smooth (fire-polish) the surface of the break by holding it in a hot flame; the bore of the opening can be reduced to any desired size by continued heating, or increased in size by manipulating with the tip of the file or with a triangular copper flanging tool or carbon cone.

b. TEST TUBES. Make a longer file cut than for glass tubing, preferably encircling the tube. A thin tube may be broken at this point by a bimanual snap. Thick tubes require additional aids to complete the break. At one point make the file nick especially deep, then touch the tube firmly at this point with the tip of a red-hot nail; a fracture should result. If the fracture is not complete, it may be led around the tube by repeatedly touching the red-hot nail tip just ahead of the fracture line on the cold tube. A heavy copper wire, curved to fit the tube and heated, is a convenient tool for this purpose. Another method is to encircle the tube with a loop of electrically heated resistance wire. The cut end of the tube should be fire-polished.

15. Bending of Glass Tubing

Holding both ends, place the tubing in the hot flame of a fish-tail burner. Rotate tube while it is heating to make the heat even on all sides. When the glass is soft, remove from flame and bend to the desired form, keeping it in that position until it has hardened. If a broad bend is desired, as in making a U bend, 10 to 15 cm of the tube should be so heated. If only a slight bend is to be made, 2 to 3 cm will suffice. At first one may have a tendency to overheat the glass and draw the two ends apart, thus distorting the shape and caliber. Also if one underheats or puts forced pressure on the bending effort, an undesirable collapse of the tubing at the bend will occur. A satisfactory bend retains the same caliber throughout the tubing. If, in working with thin tubing, the collapse at the bend cannot be prevented, seal one end of the tubing and then heat to soften it at the desired point as previously described. Then apply the mouth to the open end of the tubing while effecting the bend and make enough air pressure within the tubing to prevent the tubing from collapsing. Glass rods may be bent or pressed to any desired form. Soft glass should be annealed after working by holding it for $\frac{1}{2}$ minute to several minutes, depending on its size, in a smoky flame just under a red heat.

16. Capillary Pipettes

Glass tubing is cut to desired lengths, each end fire-polished, and put aside for future use. To form these lengths of glass tubing into pipettes a central area is heated with continuous rotation of the tube over the hot flame until soft, removed from the flame, drawn apart by pulling on the two ends, held taut until hardened, and then cut in the middle with the point of the hot flame. These are commonly called "Pasteur pipettes." The size of the resultant capillary tubing depends on the degree of heat, and the rapidity and extent of the drawing out. The tendency is to make the capillary too narrow by drawing it out too rapidly. Many laboratories keep on hand 20-cm lengths of clean sterile glass tubing with both ends rounded and plugged with cotton for use in making into pipettes as desired for special purposes.

17. Ampules

Heat a clean, sterile, cotton-plugged test tube in a narrow band about 5 cm from the mouth, with constant rotation. When the glass is dull red, remove from flame and draw the two ends about 5 cm farther apart, leaving a neck about 4 mm in diameter. When filling the ampule, be careful not to get any liquid on the neck.

To seal, heat the neck in a small, hot flame until the glass collapses, then pull out rapidly. If a strand of glass protrudes, heat it rapidly in a hot flame until it melts down to a small bead. A large bead will usually crack off on cooling. Avoid overheating, as the air inside may expand enough to blow a hole in the molten glass.

18. Blowing Bulb in Glass Tubing

Select a piece of glass tubing of the desired size and of sufficient length to allow for holding one end by hand and the other in the mouth while blowing. Seal one end. Hold the tubing over the flame, rotating with both hands, until the desired portion is well softened. Immediately place the open end of the tube in the mouth and blow up the bulb to the required size. If the bulb is to be of considerable size, some concentration of glass must be attained before the final blow; this is done by gently pushing the two ends toward each other while the middle is soft, giving an occasional slight blow to prevent collapse of the melted glass. The trouble encountered may consist of eccentric bulbs due to uneven heating, or of thin paper-shell bulbs due to overblowing without sufficient concentration of glass. Rapid intermittent puffs will aid since the thinner parts blown out first will cool first and subsequent puffs will expand the thicker parts. A test tube may be similarly handled. A terminal bulb may be made at the end of glass tubing by a one-hand manipulation and blowing.

19. Salvaging Damaged Glassware

Many pieces of damaged glassware can be saved and rendered satisfactory for further use by examining all glassware at time of washing and picking out selected damaged pieces for repair.

a. PIPETTES. Chipped tips may be smoothed with a file and trimmed to evenness in a flame; pipettes with broken tips may be drawn out and new ends prepared as described for capillary pipettes. It must be recognized that repair of a tip alters the volume and such repaired pipettes are unsuitable for exact measurements. Mark them plainly and reserve them for crude measurements. Pipettes with chipped or broken mouth parts can be saved by evening off with file and smoothing in the flame. Make the mouth part even by pressing gently on smooth, flat surface of a solid object while hot, or by cutting off a portion and polishing in flame.

b. FLASKS, BEAKERS, AND TEST TUBES. Similarly, many other pieces of glassware with chipped, cracked, or broken lips, mouth parts, or rims can be saved by removing sharp edges with a file

and fire-polishing, or by shaping with the tip of the file while the glass is hot enough to work.

Section VI. CLEANING GLASSWARE

20. Mechanical Cleaning

For ordinary laboratory use, glassware, either new or used, may be cleaned satisfactorily by scrubbing with a suitable brush in hot, soapy water. It should be rinsed in *hot* tap water repeatedly, and finally in distilled water. Cleaning is much facilitated if glassware is immersed in water immediately after use, so that organic matter will not dry on it. If glassware shows satisfactory drainage of water, as described in paragraph 21, it can usually be cleaned merely by rinsing in distilled water.

21. Chemical Cleaning

If glassware is dirty with material that cannot be removed by the process described above, it must be cleaned in one of the following solutions. If grossly dirty, it should first be cleaned with soap and water.

a. SULFURIC AND CHROMIC ACID MIXTURE.

- (1) Pour 1 liter of concentrated commercial sulfuric acid into 35 ml of a saturated aqueous solution of technical grade sodium dichromate. *Never* pour the aqueous solution into the acid. Handle with care, and avoid contact with skin or clothing. *Never* use a metal or enameled container.
- (2) Rinse the glassware with this solution, or immerse until clean; the necessary immersion may be for a few minutes or overnight. Heating the solution increases its effectiveness. Then rinse repeatedly in hot tap water until all traces of solution are removed. Finally rinse in distilled water.
- (3) In rinsing, note whether the water *completely* wets all the interior surface of the glass and runs off a thin film. If it collects in drops or patches, the glassware is not clean and the process must be repeated. When the solution weakens or turns green with use, it should be discarded. Dilution with water weakens the solution. Rinsing must be thorough, as traces of the solution render the glassware unfit for use. The first few rinsings should be made with the vessel full of water to displace

fumes. This is also true of the following cleaning solutions.

b. TRISODIUM PHOSPHATE. This is an alkaline salt that is excellent for removing greasy films from glassware. Immerse glassware in a 5 to 10 percent aqueous solution for 15 to 30 minutes. Scrub with a stiff brush; rinse thoroughly. Repeated cleaning with this agent may cause clouding of the glass. Do not employ this for glassware used for the determination of phosphorus.

c. ALCOHOLIC SODIUM HYDROXIDE. This is effective for removing tarry and greasy residues. Dissolve 120 gm of sodium hydroxide in 120 ml of water, cool, and dilute to 1 liter with 95 percent ethyl alcohol. The solution may be used hot or cold. It must be followed by thorough rinsing in tap and distilled water.

d. WETTING AND OTHER AGENTS.

- (1) Wetting agents are principally sulfonate higher alcohols, such as sodium lauryl sulfonate, and are known under the trade names of their manufacturers, such as Duponal, Nacconal, Aerosol, and Tergitol. Their efficiency as cleansing agents is due to their ability to reduce surface tension, thereby causing water containing them to wet objects more quickly and more thoroughly. This property is exerted by very dilute solutions, as little as 0.1 percent being sufficient to produce the desired results. These substances are generally used in conjunction with soap powders, the silicates, or trisodium phosphate. These products are not generally available for laboratory use because of other and more essential needs.
- (2) Although not related to the wetting agents in their action, the standard dish washing compounds (type I for hard water and type II for soft water): quartermaster items have the property of preventing the calcium and magnesium normally present in the water from reacting with soap to form the insoluble calcium and magnesium soaps that tend to form a scum on glassware.

22. Bacteriologic Glassware

Test tubes and other glassware that have contained cultures of bacteria (except pipettes) should be sterilized in the autoclave. The contents are then emptied into the sink (fluid cultures) or garbage can (agar cultures). Scrub in hot, soapy water (except pipettes) and rinse as directed in paragraph 21. If organic material has dried on the glass, it may often be loosened by preliminary boiling in soap suds for 1 hour. If not perfectly clean, glassware

must be placed in chromic acid cleaning solution as directed in paragraph 21a. The final rinsing must be thorough. Pipettes, after having been disinfected by submersion for several hours in a 5 percent solution of cresol, should be flushed thoroughly with tap water, and then with distilled water.

23. Serologic Glassware, Pipettes

Immediately after use submerge in water or, if used in handling infectious material, in a 5 percent solution of cresol. Rinse under the full force of the tap. Immerse in chromic acid solution overnight. Pour off acid solution and rinse six times under full force of the tap. Soak in tap water overnight. Rinse in distilled water, and dry.

24. Tubes

Rinse under full force of tap as soon as possible after use. Remove gross dirt (labels, clots) by soaking, and by scrubbing with a test-tube brush if necessary. Boil 1 hour in soap solution. Flasks and beakers may be scrubbed with hot suds without boiling. Rinse six times in hot tap water. Immerse in tap water overnight, rinse with distilled water, and dry. If any pieces are not perfectly clean, soak in chromic acid cleaning solution as directed in paragraph 21a.

25. Slides and Cover Slips

Used slides should first be boiled in soap suds or a 5 percent solution of sodium carbonate. Scrub with soap and water, and rinse in water under the tap. Put in chromic acid solution overnight. Pour off cleaning solution, rinse thoroughly under the tap, and drop into a beaker or wide-mouthed bottle containing 95 percent ethyl alcohol. Polish individually with a soft, lint-free cloth (not gauze). Flame slides, not cover slips. Store in a dust-proof box.

Section VII. STERILIZATION

26. Dry Heat

A temperature of 170° to 180° C. in a gas or electric oven for at least 1 hour is sufficient for the sterilization of small articles if loosely packed. A prolonged exposure to temperatures of more than 180° C. will char or burn cotton or paper. The following rules apply for routine use:

a. Sterilize glassware that is wrapped in paper or plugged with cotton by heating for a minimum of 2 hours at 170° to 180° C.

b. Sterilize glassware that is closed with glass stoppers or packed in metal containers by heating for a minimum of 2 hours at 170° to 190° C.

c. Glassware that is closely packed or in a large container must be heated for periods longer than 2 hours to insure penetration of heat to, and sterilization of the central portions. If small bits of white paper marked with dilute (10 percent) hydrochloric acid are placed inside packages or materials to be sterilized by hot air, the marks become brown or black beginning at about 165° C. If the marking on the paper has not turned brown, the article may be suspected of not having been sterilized.

27. Steam Under Pressure (Autoclave)

The standard laboratory autoclave is an unjacketed, horizontal type, set to maintain a pressure of 15 to 17 pounds. It is used for the sterilization of linens, cotton goods, rubber, glassware, and culture media that are not injured by high temperatures, and for killing old cultures. Routinely, sterilize for 15 minutes at a temperature of 120° C. (15 lb. pressure). Large packages or fluids in bulk require from 30 minutes to 1 hour. For example, 600 ml of culture medium in a 1,000-ml flask requires 30 minutes, the additional time being necessary to attain penetration of the bulky material to bring it to the sterilizing temperature; larger quantities require a still longer time.

a. Place the material in the autoclave, leave the door open, open the escape valve, and turn on the steam.

b. Close the door when the steam flows freely from the autoclave.

c. Leave the escape valve open until the steam escapes rapidly, then close, leaving a crack wide enough for a trickle of steam to escape.

d. Allow the pressure to rise slowly (not less than 10 minutes) to 15 pounds, and sterilize for the desired length of time.

e. Shut off the steam, and allow the autoclave to cool until the gauge reads zero. Do not hasten the cooling by opening the pet cock or other valve to allow the escape of steam.

f. If the autoclave is fitted with an outlet thermometer, figure the time of sterilization after the thermometer reads 120° C. (248° F.).

g. The regular use of a sterilizer control is recommended. Sealed tubes, containing certain chemicals, are suspended in tubes or flasks prior to sterilizing. These undergo physical and color

changes when conditions essential to heat sterilization have been achieved and, thus, act as a check on autoclave pressure and temperature gauges which occasionally become defective.

28. Steam Not Under Pressure

The Arnold sterilizer, or the autoclave using flowing steam without pressure (with escape vent open), may be used for materials, such as nutrient gelatin, milk mediums containing carbohydrates, that may be damaged by overheating.

- a. Place the Arnold sterilizer over a good gas burner, and bring the water in the pan to a boil.
- b. Remove the top, or the open door, and place the medium in the inner compartment; then replace, or close.
- c. Heat for 20 to 30 minutes.
- d. Leave the medium at room temperature, and repeat the procedure on the 2 succeeding days.

29. Hot Water

a. Syringes used for injections and drawing blood, and dissecting instruments are sometimes sterilized by boiling in water for 30 minutes. The addition of a small amount of sodium carbonate or borax to the water helps to prevent rusting.

b. A water bath at 56° to 60° C. may be used to sterilize vaccines if they are known to contain only non-sporulating organisms. Tubes should be immersed for an hour. Sterility should be checked by subculturing.

c. An inspissator is convenient for producing smooth slants of media containing serum or egg albumin. After tubes are placed in position, the water is heated and maintained at 75° to 80° C. until coagulation occurs. Heating is repeated for an hour on each of the 2 following days, or, following coagulation, the tubes may be autoclaved with precautions as given in paragraph 272*n*.

30. Chemicals

a. A 2 to 5 percent solution of Compound Solution of Cresol U.S.P. may be used to sterilize discarded cultures, used glassware, and rubber gloves, and for disinfecting laboratory floors and table tops.

b. Bacterial antigens and vaccines may be sterilized by the addition of 0.5 percent phenol.

c. Antisera may be preserved by addition of 50 percent glycerol, or 0.5 percent phenol; normal serum for the preparation of Loeff-

fler's medium may be preserved by the addition of 2 percent chloroform.

31. Filtration

Solutions and culture mediums that may be injured by heat sterilization, or virus-containing material, may be freed from bacteria by filtration, but it must be borne in mind that not all viruses are filtrable. Several kinds of filters have been used: porcelain (Chamberland-Pasteur), diatomaceous (Berkefeld and Mandler), and asbestos (Seitz). All are made in various sizes and of various degrees of porosity and must be selected for the purpose in mind. The porosity of Chamberland-Pasteur filters is graded from L2 (coarse) to L7 (fine); that of Berkefeld filters is indicated as V (coarse), N (medium), and W (fine); Mandler filters are marked with numbers indicating pounds of pressure required for passage of air under specified conditions; the porosity of the "E.K." pads for the Seitz filter corresponds closely to that of the Berkefeld W filter. Certain of these filters, of foreign manufacture, may not be obtainable.

a. FILTERING.

- (1) Set up the filter in a filtering flask. The stopper through which the filter stem passes should be securely tied in place, and the side arm of the flask should be plugged with cotton. Sterilize the assembled filter in the autoclave.
- (2) Attach the side arm of the flask to a suction pump in series with a manometer and trap.
- (3) Place the material to be filtered into the receptacle above the Seitz filter or into the glass mantle surrounding the Chamberland-Pasteur, Berkefeld or Mandler candle, and draw it through the filter into the flask, using a minimal amount of suction. If foaming is objectionable, and there is no contraindication to its use, a drop or two of caprylic alcohol may be added.
- (4) It is recommended that a broth culture of *Serratia marcescens* be added to the material being filtered. The passage of this innocuous and easily recognized organism through the filter indicates that the filter was defective.
- (5) With aseptic technic, remove the filtrate from the flask. Usually it is convenient to remove the stopper and the filter from the flask to a vessel in which they may be disinfected, stoppering the flask with a sterile cotton plug.

b. CLEANING OF FILTER.

- (1) If infectious material has been filtered, the filter must be disinfected before being cleaned. This may be done by boiling in water containing a small amount of sodium carbonate or trisodium phosphate or by covering the filter with 5 percent solution of phenol or cresol for at least 1 hour and then rinsing with tap water. The asbestos pads from Seitz filters are discarded after use.
- (2) After disinfection and drying, porcelain filters without metal parts may be heated red hot in a furnace to burn out organic matter, after which they are washed with distilled water to remove inorganic salts. For cleaning diatomaceous filters with metal parts there are several methods, some of which are destructive, others tedious and time-consuming. One of the best employs a 1 percent solution of potassium permanganate, which is drawn through the filter as set up (50 to 75 ml for a 1- by $\frac{3}{8}$ -inch candle). Subsequently, a 5 percent solution of sodium bisulfite is drawn through until all color has disappeared from the candle. Distilled water is then drawn through until a test of the filtrate with a solution of barium chloride solution shows no precipitate. The filter should then be dried.
- (3) Filters should be handled by the metal or glazed parts only, and should be protected from contact with grease or oil, since such substances affect the permeability, allowing passage through the filter of objects otherwise retained.

Section VIII. CARE OF LABORATORY ANIMALS

32. Types of Animals

Five types of laboratory animals in more or less common use are the rabbit, guinea pig, mouse, albino rat, and monkey.

33. Reception Quarantine

All animals received from an outside source should be isolated from 10 days to 3 weeks in previously disinfected quarters and should be found to be free from disease before mixing with regular stock.

34. Housing

Animal quarters should be kept clean, dry, and completely free from vermin. The optimum temperature for most animals is 65° to 70° F., with adequate ventilation. The standard large (10½-inch) and small (8-inch) animal jars are suitable for rats and mice; the large jar also can be used for a small guinea pig. The standard galvanized-iron animal cage (14- by 14- by 16-inch) holds one rabbit or several guinea pigs. For use in breeding rabbits or guinea pigs, larger cages or pens, preferably with outside runways, should be built. The bottom of the jar or the tray in a cage should contain an absorbent bed material, such as wood shavings; hay or straw may be used in large breeding cages. The quarters should be cleaned and the bedding renewed twice per week.

35. Rabbits

a. FEEDING. The diet recommended consists of commercial "Rabbit Pellets," supplemented once or twice each week with the feeding of green-stuff, such as carrots, lettuce, and celery tops. A diet consisting of equal parts of oats, wheat, and barley, plus 10 percent of legume, soybean, or linseed meal, is suitable. Alfalfa or timothy hay serves both for food and bedding. Plenty of water and a small piece of rock salt should be kept in the cage.

b. DISEASES.

- (1) Coccidiosis, an intense and fatal enteritis, is the most serious disease. There is no effective treatment. New rabbits should be quarantined for several days before adding to the stock, in order to minimize the possibility of introducing the disease.
- (2) Ear mange is caused by a mite; it can be cured by the local application of a parasiticide.
- (3) Snuffles is a coldlike disease caused by a filtrable virus. Infected rabbits should be isolated until 3 weeks after recovery.

c. BREEDING. Keep one male (buck) for each 8 to 10 females (does). Does are ready for mating at the age of 10 months and may be bred every three months thereafter (4 litters per year). A record of each date of breeding should be kept; the period of gestation is 31 days, and 2 or 3 days before the expected arrival of the litter the doe should be placed in a small breeding box, with an ample supply of bedding. Wean young after 8 weeks and separate sexes.

36. Guinea Pigs

a. **FEEDING.** The feeding is the same as that for rabbits, except that they must have a supplementary feeding of greenstuffs at least twice a week to supply vitamin C.

b. **DISEASES.**

(1) *Salmonella* infections, due chiefly to *Salmonella typhimurium* and *S. enteritidis* are the most dangerous of the common diseases. The best method of control is to kill all potentially infected animals, to sterilize the room and cages, and to obtain new stock.

(2) Vitamin C deficiency is caused by lack of sufficient greenstuffs in the diet. It is characterized by coarse hair and a mangy appearance. It is transmissible to the young through the mother. Treatment consists of an improved diet.

c. **BREEDING.** Use colony breeding, with four or five females in a cage with one male. The duration of pregnancy is 63 days. The young should be weaned and the sexes separated when they are 4 or 5 weeks old.

37. Mice and Rats

Several different mouse strains are used, such as white mice, Swiss mice (also white), and C 57 strain (black).

a. **FEEDING.** Commercial dog- or fox-food (dry type) furnishes an ample, balanced diet for growth and breeding. There must be a supply of fresh, clean water in the cage at all times. Mice also do well on simpler diets, such as the mixed-grain diet for rabbits, and dry bread with water or skimmed milk, with the addition of cod-liver oil once a week.

b. **DISEASES.** *Salmonella* infections (mouse typhoid), caused by the same organisms as are infectious in guinea pigs, are frequent and dangerous. To control, all infected stock should be destroyed, the room and cages sterilized, and fresh stock obtained.

c. **BREEDING.** Use colony breeding, with four or five females to one male. The period of gestation is 21 days; when well-advanced pregnancy is observed, the female should be placed in an individual jar. After 21 days the young should be isolated, and the mother returned to the breeding cage. The young are fed the same as the adults, with the addition of evaporated milk to hasten growth.

d. **ALBINO RATS.**

(1) *Feeding.* Rats are fed the same as mice.

(2) *Diseases.* If the cages are kept clean and an ample diet is provided, rats are extremely resistant to disease.

- (3) *Breeding*. Young females are ready for breeding when 4 months old. Use the colony method, with four females and one male in a cage. The duration of pregnancy is 22 days. It is not necessary to remove the pregnant female from the breeding cage. The young should be weaned and the sexes separated when they are 21 days old.

38. Monkeys

a. FEEDING. Monkeys do well on dry dog food and canned tomatoes, with an occasional feeding of fruits and nuts (oranges, apples, bananas, peanuts, sunflower seeds).

b. DISEASES.

(1) Pneumonia, which is usually fatal, is the most frequent disease.

(2) Miliary tuberculosis occasionally occurs. This also is fatal.

c. BREEDING. Breeding in captivity in small laboratories is not practical.

Section IX. ANIMAL EXPERIMENTATION

39. Purposes

Animal experimentation is used for the following purposes:

a. General study of pathogenic micro-organisms.

b. Virulence determinations of specific organisms in certain animal species.

c. Identification of some species of organisms by the production of characteristic lesions.

d. Isolation of organisms that are not readily grown initially on ordinary culture mediums.

e. Maintenance of bacteria or viruses that cannot be kept on ordinary culture mediums.

f. Studies of immunity.

40. Animals Used

a. Guinea pigs, rabbits, white mice, and white rats are routinely used.

b. Horses, monkeys, fowl, canaries, hamsters, and ferrets are occasionally used.

41. Methods

The methods of inoculation are as follows:

- a.* Subcutaneous (under the skin).
- b.* Intracutaneous (into the skin).
- c.* Intramuscular (into a muscle).
- d.* Intravenous (into a vein).
- e.* Intraperitoneal (into the abdominal cavity).
- f.* Intrapleural (into the pleural cavity).
- g.* Intracerebral (into the brain).
- h.* Subdural (into the membranes of the brain or spinal cord).
- i.* Per os (by mouth).
- j.* Per rectum (by rectum).
- k.* Miscellaneous (other channels of application).

42. Subcutaneous Inoculation of Guinea Pigs

- a.* The usual site is the abdominal wall or inner thigh.
- b.* Remove the hair from the area by pulling it out, small tufts at a time, or by clipping or shaving.
- c.* Sterilize the skin site with tincture of iodine, alcohol, or another antiseptic.
- d.* Take up the inoculum in a glass syringe, fitted with a fine gauge needle.
- e.* Plunge the needle through skin while it is raised by grasping the neighboring skin with the fingers, to avoid penetrating the muscle.

43. Intraperitoneal Inoculation

- a.* The site and inoculum are prepared as in paragraph 42.
- b.* Plunge the needle through abdominal wall by two movements:
 - (1) Through the skin in an oblique direction.
 - (2) Through the muscle and peritoneum by a short vertical stab.
- c.* Exercise care not to puncture the intestine.

44. Intravenous Inoculation of Rabbits

- a.* The veins along the margins of ears are the usual sites of injection.
- b.* The hair over the site is shaved, and the animal is held head downward for a short time.
- c.* The rabbit is held by an assistant, or is placed in an animal box with its head out.

d. If desired, the ear may be placed over an electric light bulb, with the base of the ear slightly compressed (both devices make the veins more prominent).

e. Holding the tip of the ear with the left hand, the operator inserts the needle with the right hand, the needle entering the vein nearly parallel to its course.

f. The injection is started slowly, until it is certain that inoculum is entering vein; if it passes along course of vein by perivascular leakage, another puncture must be made.

45. Mouse Inoculation

a. INTRAPERITONEAL.

(1) The mouse is held by the back of the neck with the thumb and forefinger of left hand, and the root of the tail is held between the little finger and palm, the mouse resting ventral side up in the palm of the hand.

(2) Needle puncture is made into abdomen by a quick, short jab.

b. SUBCUTANEOUS. Injection is made under the skin near the base of the tail.

c. INTRAVENOUS. Injection is made with a fine needle into a vein of the tail.

46. Bleeding of Animals

a. RABBIT. To obtain a few milliliters of blood from the ear, proceed as follows:

(1) The ear is shaved and immersed in warm water (to expand the vessels).

(2) A thrust is made into a vein with a broad, cutting Hagedorn needle.

(3) The blood is caught in a glass tube.

b. RABBIT OR GUINEA PIG. To obtain blood from the heart, proceed as follows:

(1) The animal is held (with or without anaesthesia) on its back, either by an assistant or on an animal board.

(2) The left anterior chest is shaved and painted with iodine.

(3) A glass syringe fitted with a 22-gauge, 2-inch needle is used.

(4) The needle is passed downward into the heart through the third intercostal space, close to the left side of the sternum. Slight suction is made by the syringe plunger until blood appears.

- (5) The needle is quickly removed after sufficient blood is withdrawn.
- (6) The animal is restored to its cage for several weeks rest before reuse.

c. **RAT AND MOUSE.** To obtain blood from tail, proceed as follows:

- (1) The animal is placed in a small box, with its tail out and held.
- (2) The tip of the tail is clipped off with scissors.
- (3) A stripping movement starting at the base of the tail produces a few drops of blood at the tip.
- (4) The flow of blood can be stopped by cauterizing with a hot spatula.

d. **HORSES AND SHEEP.** To obtain venous blood, a large needle is inserted into the external jugular vein, which runs from a line just behind the angle of the lower jaw to the sternoclavicular junction.

47. Blood Handling for Various Purposes

a. **WHOLE BLOOD FOR CULTURE MEDIUM.** The blood is placed at once into the medium.

b. **CITRATED BLOOD FOR CULTURE MEDIUM.** The blood is drawn, or placed at once, into a sterile flask containing an anticoagulant, such as enough sodium citrate to give a 0.25 percent concentration after the blood is added.

c. **DEFIBRINATED BLOOD FOR CULTURE MEDIUM OR SERUM.** The blood is drawn, or placed at once, into sterile flasks containing glass beads, and defibrinated by several minutes of gentle shaking; it may then be left at ice-box temperature until used or separated.

d. **SERUM FOR SEROLOGIC TESTS.** The blood is drawn or placed into sterile tubes or flasks, and allowed to clot in the ice box; the serum is removed after clot has contracted. Centrifugation may be required to remove the cells.

48. Post Mortem Examination of Small Animals

a. An autopsy should be performed as soon after death as possible, especially if cultures are to be made.

b. The necessary instruments are as follows: 4½-inch scissors, 1 point sharp (for guinea pigs and rabbits); scissors, straight strabismus and curved iris scissors (for mice); scalpels; tissue forceps; hemostatic forceps; autopsy board; thumb tacks; and special instruments, such as curettes. (The instruments must be sterile if cultures are to be made.)

c. Sterilize the surface of small animals by immersing the entire animal, except nose and mouth, in a 3 percent solution of cresol; sterilize the desired portion of larger animals with cresol or iodine after placing on the autopsy board.

d. Tack down small animals through each foot to a wooden board; larger animals are tied down on an autopsy pan or board by the feet, with the ventral side up.

e. Snip skin of abdomen in the pubic region, insert the blunt point of a pair of scissors, and make a cut along the median line to the lower jaw; cut the skin on each side at a right angle to the median cut, and continue these cuts along the legs to expose the inguinal and axillary lymph glands.

f. Using tissue forceps and the blunt point of a pair of scissors, separate the skin from the abdomen and retract; examine the exposed tissue and lymph nodes for abnormalities.

g. If sterility is desired, paint the abdominal wall with iodine or wash with acetone-alcohol, or both, and use a separate set of sterile instruments for further examination.

h. Open the abdomen by making a median incision from the genital region to the diaphragm, followed by terminal lateral cuts; lay back the abdominal wall as flaps on each side of animal.

i. Examine the abdominal organs for gross lesions, prepare smears, and make cultures.

j. To enter thorax, first cut the diaphragm free from its anterior attachment to the thoracic cage; then cut the ribs on each side, starting with the floating ribs laterally and proceeding at an angle up to the suprasternal notch; remove this section or lay it back to expose the thoracic cavity. Study the heart and lungs for presence of pathologic lesions, and prepare smears and cultures.

k. Place the organs or tissues to be given histopathological study in a bottle of 10 percent formalin or Zenker's fluid.

l. Remove the animal from the board, wrap it firmly in paper, and dispose of it as circumstances permit, preferably by immediate incineration.

Section X. COLLECTION AND PREPARATION OF HUMAN BLOOD SAMPLES

49. Purpose

Blood is obtained by venipuncture for making blood cultures, serologic tests, blood-group determinations, chemical tests, and many of the special hematologic examinations described in chapter

2. It is also suitable for cell counts, although if this is the only examination required, finger puncture suffices.

50. Venipuncture

a. FOR BLOOD CULTURE.

- (1) The following materials are required: A sterile, 10-ml Luer syringe and a sterile 20-gauge needle; sterile cotton or gauze pledgets; tincture of iodine and 70 percent ethyl alcohol; tourniquet; an alcohol lamp or Bunsen burner; and a flask of suitable culture medium or a small flask or large test tube containing 2 to 5 ml of a 1 percent solution of sodium citrate in physiologic salt solution.
- (2) The procedure is as follows:
 - (a) Have the patient or subject lie down with the arm extended, or sit with the arm supported on a small table, the elbow being extended.
 - (b) Select a larger vein, preferably in the bend of the elbow. If no suitable vein is visible, apply the tourniquet temporarily, and have the patient clench fist. In plump arms, one can frequently feel the vein more distinctly than one can see it. If no vein can be located in this region in either arm, it is usually possible to find a vein lower in the forearm or on the back of the hand. Here, however, the skin is tougher and more sensitive and it is more difficult to puncture the vein without transfixing it completely.
 - (c) Thoroughly cleanse the skin over the vein and surrounding area with a pledget soaked in alcohol.
 - (d) Paint the area with tincture of iodine.
 - (e) Light the alcohol lamp or Bunsen burner.
 - (f) Unwrap the syringe, and insert the plunger in the barrel without touching the inside of the barrel or the shaft of the plunger with the fingers.
 - (g) Remove the cotton plug from the needle tube, and flame the mouth of the tube.
 - (h) Insert the neck of syringe into the mouth of the tube, and tilt the tube so that the needle slides down over the neck.
 - (i) Remove the syringe and needle from the tube, and set the needle firmly on the neck with the beveled edge up when the syringe is held so that the graduations are visible. Remove the stylet. *Take care to touch only the hub of the needle.*

- (j) Flame the mouth of the tube that contained the needle, and momentarily, the needle point. Cover the needle with the flamed tub, and set aside until preparation of the arm is completed.
 - (k) Apply the tourniquet above the elbow just tightly enough to distend the vein. Be sure the pulse is not obliterated. Have the patient clench the fist, if necessary.
 - (l) Remove the iodine with a pledget soaked in alcohol.
 - (m) Puncture the skin with the needle, beveled edge up, a little to one side of the vein and parallel to it; then enter the vein from that side, about 1 cm above the skin puncture.
 - (n) Aspirate gently until the desired amount of blood has been obtained, loosen the tourniquet, and have the patient open the fist.
 - (o) Withdraw the needle quickly, and press an alcohol-soaked pledget firmly over the puncture. Have the patient hold the pledget tightly in place with the arm held upright.
 - (p) Remove the cotton plug from the flask or tube, holding it in fingers in such a way that the portion fitting within the neck does not touch anything. Flame the neck thoroughly.
 - (q) Insert the needle into the flask or tube, and discharge the blood by pushing the plunger. Withdraw the needle from the flask without touching the sides of the flask.
 - (r) Reflame the neck, and replace the plug.
 - (3) If the blood has been put directly into a flask of culture medium, it should be well mixed by gently twirling the flask, taking care that the contents do not slop or spatter against the plug. It should then be put in the incubator without delay. If the blood has been put into a tube containing citrate solution, it should be well mixed, taken at once to the laboratory, distributed into the proper mediums, and put in the incubator.
- b. FOR OTHER PURPOSES.
- (1) For most examinations sterile blood is not required if the examination is made immediately, and the syringe can be dispensed with, using only the sterile needle. The procedure is exactly as given above. It is convenient to hold the needle tightly between the thumb and index finger at the hub. Grasp the tube which is to receive the blood with the third and fourth fingers, holding it so that the hub of the needle is just within the mouth of the

tube. This is much easier to accomplish if the patient's arm is allowed to hang down. (See fig. 1.)

- (2) A syringe may be used to obtain blood for any purpose, following the directions given for blood cultures. The needle and syringe must be sterile and dry, or rinsed with sterile physiologic sodium chloride solution, to prevent hemolysis, which would ruin the specimen for many purposes. Remove the needle from the syringe, and expel the blood gently into the tube or bottle to avoid mechanical injury to the cells.

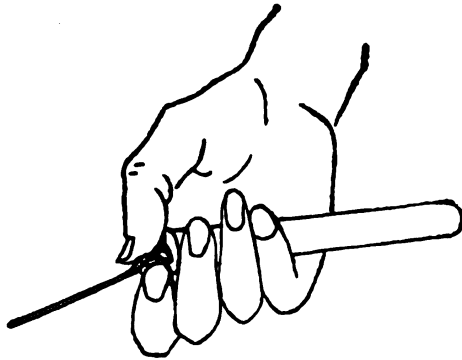


Figure 1. Venipuncture (method for holding needle and test tube when collecting blood directly from vein).

- (3) The handling of specimens varies according to their eventual dispositions.
 - (a) If serum is required, as for serologic and many chemical tests, the blood should be collected in any empty, chemically clean test tube, which need not be sterile unless the specimen is to be preserved. This should be allowed to stand at room temperature until clotting has occurred. It should then be placed in the ice box until used.
 - (b) If whole blood or plasma is required, as for chemical tests, the blood is usually collected in a tube containing potassium or lithium oxalate (2 to 3 mg for each ml of blood). Other anticoagulants are required for some determinations. The tube should be twirled so that the blood is thoroughly mixed with the anticoagulant. If the analysis is not carried out at once, the tube should be put in the ice box until used.
 - (c) If sugar or inorganic phosphorus is to be determined, the blood should be chilled by placing the tube in ice water before putting into the ice box, unless precipi-

tation of the protein is carried out immediately.

- (d) If blood is to be used for cell counts, for the determination of carbon dioxide combining power or content, or for chloride, avoid stasis as much as possible. Apply the tourniquet as lightly and briefly as possible, and release it as soon as the needle is in the vein. Use a syringe, if available, rather than an open needle.
- (e) If the blood is to be used for particular chemical tests, the special precautions listed in chapter 6 should be observed.
- (f) Take the blood to the laboratory promptly for examinations.

51. General Precautions

a. To avoid infection, which is due to carelessness, sterilize the skin carefully, be sure that the needle and syringe are sterile, and do not allow the tip or shaft of the needle to touch anything that is not sterile before obtaining the blood.

b. Inspect—*do not touch*—the point of the needle to be sure that the tip is sharp and that the margins of the beveled edge are smooth. If the tip is dull, or the margins jagged, this causes unnecessary pain and is likely to tear the wall of the vein.

c. Avoid injury to the vein, which may result in leakage of blood into the tissues (hematoma, blood tumor) or in the formation of a blood clot within the vein. This is usually due to use of a dull needle or of too large a needle in a small delicate vein, roughness in inserting the needle, twisting the needle while it is in the vein so that the point scratches or cuts through the wall, or failure to release the tourniquet before withdrawing the needle and to apply adequate pressure after withdrawal.

d. Never pass the stylet through the needle while it is in the vein. This may dislodge a clot into the circulation and cause serious injury to the patient. If the blood clots in the needle and the flow stops, loosen the tourniquet, withdraw the needle, and repeat the attempt on the other arm, using a new needle.

e. Avoid causing unnecessary pain. Have the subject lie down or sit down comfortably, use a sharp needle, and avoid excessive slowness in making the puncture.

52. Care of Needles and Syringes

a. As soon as the blood specimen has been obtained, shake as much of the blood from the needle as possible, and drop it into a beaker of cold water to luke the blood. On returning to the labora-

tory, flush the needle with cold water and pass a stylet through it to remove any clots that may have formed. Dry by forcing alcohol and then ether through the bore. Never put a needle away wet, as it will rust and be damaged.

b. Replace the stylet with the loop of the wire outside the point to protect the latter. Slide the needle into a Wasserman tube, plug the tube with cotton, and sterilize it by dry heat or in the autoclave.

c. Sharpen the needle if necessary. This is best done on the finest grade of emery cloth stretched on a flat surface, or on a fine oil stone, finishing on a fine blue water stone. Even the finest grades of emery or carborundum cloth leave a slight saw edge that may cause unnecessary pain and trauma to the tissues.

d. Immediately after expelling blood from the syringe, remove the plunger. As soon as possible wash the syringe thoroughly with cold water, and lay it aside to dry with the plunger removed. Never put a syringe away with the plunger in the barrel, as it is likely to become stuck and impossible to remove. If the plunger becomes "frozen," plunging the syringe in hot water may expand the barrel sufficiently to release the plunger. Otherwise fill the space between the neck of the syringe and the head of the plunger with cold water by injecting it with another syringe filled with a fine needle, and soak the syringe in cold water for a few days, then the plunger can usually be loosened. Never use force; it will merely break the glass.

e. To sterilize, wrap the plunger and barrel separately in gauze, with an outer wrapping of heavy paper if hot air is used, or of muslin if it is to be autoclaved. Secure the wrapping with a turn or two of ordinary twine, tied in a slip knot to expedite unwrapping. An alternative method consists of placing assembled syringes in cotton-plugged test tubes of proper size; these are sterilized in the hot-air oven.

Section XI. MISCELLANEOUS PROCEDURES: USEFUL LABORATORY RECIPES AND METHODS

53. Acidproofing Wood Surfaces

a. REAGENTS.

Solution No. 1:

Aniline	100 ml
Hydrochloric acid (concentrated)	100 ml
Water	1,000 ml

Solution No. 2:

Potassium dichromate	100 gm
Water	1,000 ml

b. APPLICATION. For satisfactory results the wood surface should be free from varnish, wax, or paint. Apply solution No. 1 and then solution No. 2, without waiting for the former to dry. Both solutions are applied continuously until a good black color is obtained. Then the surface should be washed with soap and water and allowed to dry thoroughly. Finally, a coat of wax is applied.

54. Removing Stains

a. Mortars in which stains have been ground, and bottles containing dried stains, may be cleaned by a brief soaking in concentrated sulfuric acid. This is poured back into a bottle for repeated use. The mortars or bottles are then washed out with water. Containers stained with eosin should be soaked in a 5 percent solution of sodium carbonate for a few minutes and then washed out with water.

b. Most bacteriologic stains can be removed from the hands by washing with acid alcohol (ethyl alcohol containing 2 or 3 percent by volume of concentrated hydrochloric acid), and then washing with soap and water. For cleansing fabrics, 10 percent by volume of acetic acid in alcohol should be used, followed by rinsing with large amounts of water.

c. Iodine stains are best removed with a solution of sodium thiosulfate, followed by generous rinsing.

d. To remove grease spot from cloth or leather, carbon tetrachloride usually suffices, and is noninflammable. It should be used in a well-ventilated place, and getting the tetrachloride on the skin and breathing its fumes should be avoided. Carbon tetrachloride causes degeneration of the liver if absorbed by skin or lungs in appreciable amounts.

e. For the removal of blood stains from clothing, 3 percent solution of hydrogen peroxide is useful.

55. Loosening Stopcocks and Stoppers

The freezing of glass stopcocks and stoppers is a frequent source of annoyance and loss in the laboratory. Much of it can be prevented by the use of appropriate lubricants and by preventing the prolonged action of alkali on ground-glass joints. Most frozen joints can be loosened by appropriate means. The process requires patience. "Strong arm" methods usually result in breakage and the loss of the apparatus.

a. COMMERCIAL MECHANICAL DEVICES. There are a number of excellent devices on the market for loosening glass joints. Since

they are somewhat expensive, it is usually worthwhile to purchase them only if they can be used by a considerable group of laboratories. Their successful use is based on the *gradual* application of pressure or tension to the glass joint, care being taken that the devices are not allowed to slip and fracture the glass.

b. LABORATORY-MADE MECHANICAL DEVICES. By a study of the principles on which the commercial devices operate, it is sometimes possible to improvise a mechanical device that will serve the same purpose. For example, a piece of wood may be drilled and cut in such a fashion as to furnish support for a glass joint, which is inserted in a vise and gentle pressure applied to the protruding end of a stopcock. Glass stoppers in bottles can often be loosened by gentle tapping with the butt of a hammer or similar tool.

c. USE OF HEAT. The proper application of heat alone will loosen many frozen glass joints. Chill the joint in an ice bath, then immerse it suddenly in warm or hot water. This will cause the exterior portion of the joint to expand away from the stopcock or stopper, so that the latter may be loosened by hand or by other means.

d. CHEMICAL METHODS. Ground-glass joints frozen by contact with alkali can frequently be loosened by immersion in a dilute (about 5 percent) solution of acetic or hydrochloric acid, which is brought to a boil, then allowed to cool so that the dilute acid penetrates the joint as it contracts. It may be necessary to heat and cool several times, and in stubborn cases, several days may be required. If a joint contains lubricant or other organic material, it should be heated and cooled in the same fashion in a bath containing about 25 percent of glycerol (glycerin) by volume, the glycerol serving to soften or dissolve the organic material. Large pieces of apparatus, such as burettes, should be supported by means of a clamp or a ring on a stand during the process.

56. Stopcock Lubricants

a. GLYCEROL. For sealing ground-glass joints and stopcocks to prevent leakage of ethyl ether, petroleum ether, or any other fluid insoluble in it, glycerol (glycerin) is extremely satisfactory. It also prevents sticking.

b. STOPCOCK GREASE. Melt 2 parts of paraffin and 4 parts of liquid petroleum together, then add slowly 1 part of pure gum rubber cut in small pieces. Stir while heating until a smooth paste is formed. Be careful not to burn the rubber.

c. OTHER LUBRICANTS.

- (1) Mix together 100 gm of petrolatum and 10 gm of raw crepe rubber. Keep in an oven at 125° to 150° C. for several days until the paste is smooth.
- (2) Mix 2 parts of ordinary rubber cement and 1 part of liquid petrolatum. Heat on a water bath until the solvent from the rubber cement is driven off.

57. Cements and Adhesives

a. **ACID-RESISTING CEMENT.** Mix sodium silicate and asbestos powder to make a thin paste. If allowed to dry for 1 day, this cement will resist the strongest acids.

b. **VACUUM WAX.** For ordinary vacuum seals and for vacuum distillations in which the temperature does not get too high, to make an excellent wax melt together equal parts of white wax, U.S.P. and resin. This wax is pliable and is easily removed by using hot water.

58. Label Protection

Labels may be protected by painting with collodion or melted paraffin.

CHAPTER 2

EXAMINATION OF BLOOD

Section I. CLINICAL PROCEDURES

59. General

a. PRODEDURES. The examination of the blood yields valuable facts concerning the patient. This information can be summarized as follows:

- (1) The presence of anemia (fewer than a normal quantity of red corpuscles) or polycythemia (greater than a normal quantity of red corpuscles). This can be ascertained by any one of the following:
 - (*a.*) Hemoglobin level. (See par. 61.)
 - (*b.*) Red-cell count. (See par. 62.)
 - (*c.*) Volume of packed red cells (hematocrit). (See par. 71.)
- (2) The presence and intensity of certain types of infections and certain other disorders can be ascertained by determining the—
 - (*a.*) Total leucocyte count. (See par. 63.)
 - (*b.*) Differential leucocyte count. (See par. 67.)
 - (*c.*) Sedimentation rate. (See par. 74.)
- (3) Abnormalities in the coagulation of the blood can be ascertained by determining the following:
 - (*a.*) Bleeding time. (See par. 77.)
 - (*b.*) Platelet count. (See par. 76.)
 - (*c.*) Coagulation time. (See par. 78.)
 - (*d.*) Clot-retraction time. (See par. 79.)
 - (*e.*) Prothrombin time. (See par. 80.)
- (4) Differentiation of various types of anemia is aided by a study of the following:
 - (*a.*) Morphology of the red cells in the stained blood smear. (See par. 68.)
 - (*b.*) Calculation of the mean volume and the hemoglobin content of the red cells. (See par. 72.)
 - (*c.*) Reticulocyte count. (See par. 75.)
 - (*d.*) Icterus Index. (See par. 73.)
 - (*e.*) Fresh blood smears. (See par. 69.)
 - (*f.*) Red-cell fragility test. (See par. 81.)

- (5) Parasites in the blood may be recognized by the use of appropriate methods. (See par. 69 and ch. 13.)

b. **IMPORTANCE OF ACCURACY AND TECHNICAL HONESTY.** The value of any of these procedures is lost if they are not performed accurately. If directions are followed carefully, however, most of these examinations can be made with reasonable accuracy. Errors can and do arise, nevertheless, and serious harm to the patient can be avoided if they are discovered promptly and corrected. Such technical honesty is fundamental.

60. Finger Puncture

a. **MATERIALS.**

- (1) A puncture instrument with a sharp cutting edge, Hagedorn needle or Bard-Parker blade. When these instruments are not available, the end of a broken glass slide can be used. Carefully clean the cutting blades after use and sterilize by autoclaving or boiling.
- (2) Gauze; ethyl alcohol (70 percent) or acetone-alcohol (equal parts).
- (3) Clean pipettes and chemically clean slides.

b. **PROCEDURE.**

- (1) Rub the finger briskly or place the hand in warm water to promote blood flow (an ear lobe may be used).
- (2) Clean the finger with alcohol or acetone-alcohol, and dry. If the fingertip is wet, the blood will not form a round drop.
- (3) Hold the side of the patient's finger between your thumb and index finger. Puncture the finger with a firm, quick stroke deep enough for the blood to flow immediately. Avoid squeezing the finger close to the site of puncture because this forces tissue juices into the cut and dilutes the blood.
- (4) Wipe off the first drop with dry gauze.
- (5) Allow a large drop to collect before touching a blood pipette or slide to the drop. For smears on cover slips small drops of blood are used.

61. Hemoglobin Content

a. **GENERAL COMMENTS.**

- (1) The measurement of the hemoglobin in the blood is the simplest method of determining the presence of anemia or polycythemia; it can be done quickly with a minute amount of blood. The accuracy of the result, however,

depends on the method used. More precise procedures are red-cell counts (par. 62) and the hematocrit (par. 71). The former has the disadvantage that extensive skill and training are necessary to insure accurate counts, and the latter that blood must be obtained from a vein and that a centrifuge is required.

- (2) The quantity of coloring matter (hemoglobin) in the blood can be measured in many different ways. The accuracy of the various methods differs greatly and consequently the method used should be stated. The use of crude methods, such as the Tallquist scale, is condoned only because under certain conditions nothing better is available.
- (3) It has long been the custom to express hemoglobin in percentage, the object being to state the hemoglobin value in proportion to normal. This object cannot be fulfilled because the amount of hemoglobin varies in normal individual with age, sex, climate, and other factors, and therefore no value can be arbitrarily fixed as the equivalent of 100 percent hemoglobin. Like other chemical constituents of the blood, the hemoglobin should be expressed in grams per 100 ml of blood. For the calculation of color index it is necessary, however, to choose some value in grams of hemoglobin which may be used as the equivalent of 100 percent hemoglobin. Since in the calculation of color index 5,000,000 red cells is used as 100 percent red cells, and in order that the color index of normal blood be found at 1.0, it is necessary to choose for 100 percent hemoglobin the value which corresponds, on the average and in the male and female, to 5,000,000 red cells. This value (the "hemoglobin coefficient") is 14.5 gm. Various other values, however, have been used as the equivalent of 100 percent hemoglobin (see details below concerning each instrument). It is, therefore, necessary to state hemoglobin both in grams and in percentage.
- (4) In general, methods of measuring hemoglobin can be classified as direct and indirect. Direct methods measure the red color of blood; for the indirect procedures hemoglobin is mixed with $N/10$ hydrochloric acid to make a brown liquid, acid hematin. The latter is the preferable procedure because many persons are unable to match red. It is important to know, however, that acid hematin gradually increases in color intensity. It is necessary, therefore, to make readings at the same time interval

after dilution. The desirable time is stated in the instructions given for each instrument.

b. TALLQUIST METHOD.

(1) Materials.

- (a) Finger-puncture equipment.
- (b) A Tallquist scale, which is a paper strip with red bands, each colored to represent from 10 to 100 per cent hemoglobin content on test paper.
- (c) Absorbent paper (supplied in the book with the scale).

(2) Procedure.

- (a) Blot a drop of blood with a sheet of the absorbent paper, setting the paper aside for a moment until the sheen has disappeared.
- (b) Match the test spot against the color standard. Use a white background, in daylight if possible. Note the figure of the color band similar to the color of the test spot.
- (3) *Report.* Reports are expressed in percentages; for example, "90 percent (Tallquist)." Since the method reveals only gross changes it is useless even to attempt to express the result in grams.

c. SAHLI METHOD. The Sahli hemoglobinometer recommended consists of a square calibrated tube that comes in direct apposition with a brown-glass comparator rod; a pipette for measuring the proper amounts of blood is also supplied. This is the most generally available instrument of reasonable accuracy.

(1) Procedure.

- (a) The calibrated tube is filled with $N/10$ hydrochloric acid to about the "20" mark.
- (b) The pipette is filled with blood to the "20 cu mm" mark. *It is important that all blood adhering to the outside of the pipette be removed before the dilution is made.*
- (c) The pipette is carefully introduced into the calibrated tube and passed to the bottom, into the acid. The blood is then *slowly* expelled, care being taken to form no bubbles. When all the blood has been expelled the pipette should be rinsed twice with distilled water, the contents being expelled into the calibrated tube.
- (d) After waiting for a fixed interval (5 minutes), distilled water is added gradually until the shade agrees with that of the standard brown-glass rod. In mixing the contents of the calibrated tube, the fluid should never be permitted to touch the finger, or a loss of

fluid occurs with resulting inaccuracy in the ultimate reading. The stirring rod supplied should be used.

- (2) *Report.* The amount of hemoglobin is determined by the final volume in the calibrated tube. The report should be expressed in grams and percentage; for example, "13.0 gm, 90 percent (Sahli)"; 14.5 gm equals 100 percent.

d. **OTHER COMMONLY USED METHODS.** Directions for the use of other types of hemoglobinometers will be found with the instruments. The standards for some of them are as follows:

Fisher electrohemometer: 15.6 gm equals 100 percent hemoglobin.

Haden-Hausser hemoglobinometer: 15.4 gm equals 100 percent hemoglobin.

Newcomer hemoglobinometer: 16.9 gm equals 100 percent hemoglobin.

Dare hemoglobinometer: 16.0 gm equals 100 percent hemoglobin.

e. **COPPER SULFATE METHOD** (Phillips, VanSlyke, et al). In this recently devised method, the hemoglobin concentration is calculated from the specific gravity of the whole blood and plasma, separately determined. (See par. 215.)

62. Red-Cell Counts

a. **MATERIALS.** The following materials are required:

- (1) *Diluting pipette.* This often has a red bead in the bulb to make it quickly recognizable. The Thoma pipette is marked in graduated lines along the capillary bore. (See fig. 2.) The fifth graduation from the tip is marked

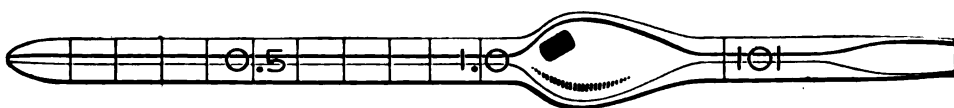


Figure 2. Red-cell pipette.

"0.5," the tenth "1.0"; above the bulb is a line marked "101." In this pipette, if blood is drawn to the 0.5 mark and the diluting fluid to the 101 mark, the dilution is 1:200.

- (2) *Counting chamber.* The Levy chamber with the improved Neubauer ruling is the supply table item of issue. There are other types of ruling and several kinds of chambers, all similarly used. The chamber is a thick glass slide with two central platforms; on the surface of each is engraved a series of rulings. The slide platforms

on which the special cover glass fits are exactly 0.1 mm higher than the central platforms. When the cover slip is in place there is a space 0.1 mm deep, each ruled area having a surface area of 9 sq mm. The four larger corner squares outside the triple-ruled lines (1, 2, 3, and 4 in fig. 3) are each subdivided into 16 smaller squares. The central square is divided by triple lines into 25 small squares each of which contains 16 smaller squares, making a total of 400 squares. Each small square therefore represents 1/400 sq mm.

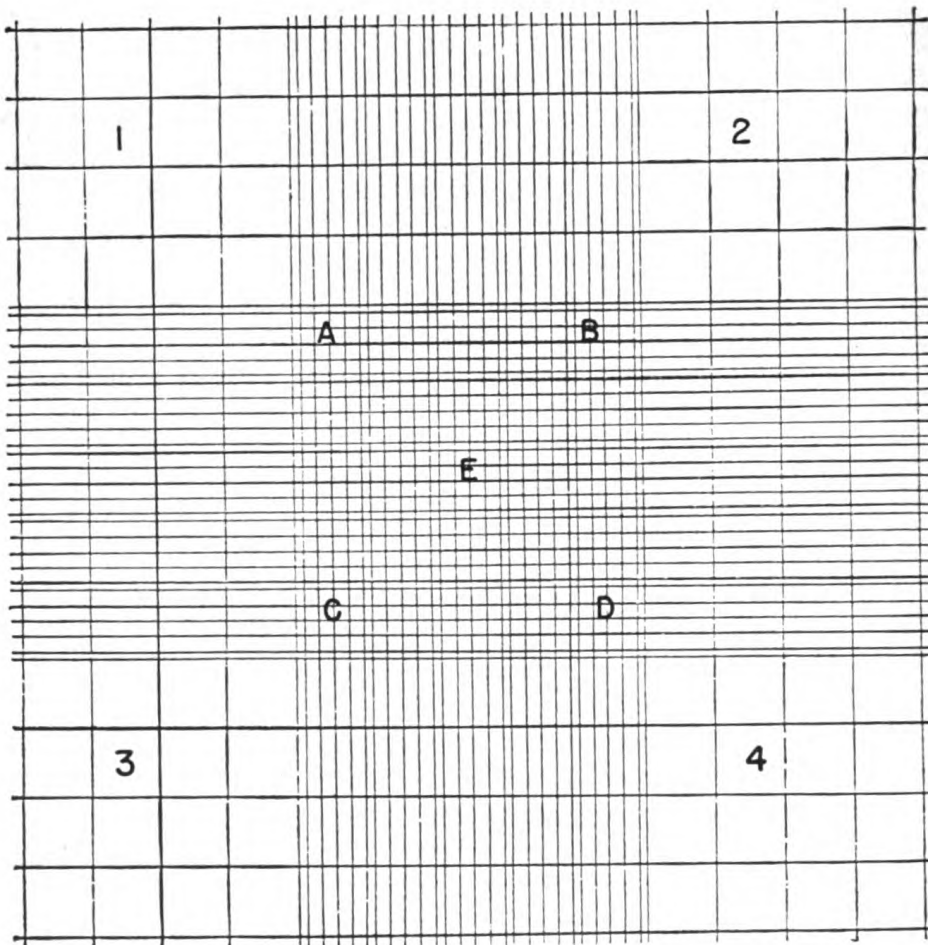
- (3) *Diluting fluid* (Hayem's solution). This is prepared as follows:

Sodium chloride	-----	1.0 gm
Sodium sulfate	-----	5.0 gm
Mercuric chloride	-----	0.5 gm
Distilled water	-----	200 ml

- (4) *Finger* — puncture equipment (or a sample of oxalated venous blood.)
 (5) Water, alcohol, and ether (for cleaning pipettes).
 (6) Microscope.

b. **CLEANING OF COUNTING CHAMBER AND PIPETTE.** All pipettes and counting chambers should be clean and dry before using and should be cleaned immediately after using. Avoid harsh rubbing or strong solutions on the counting chamber. Remember that the depth of the prepared chamber is accurate *only* if the chamber, shoulder supports, and cover glass are perfectly clean.

- (1) *Counting chamber.* Cleanse the surface of the counting chamber with water. Dry with soft gauze and lens paper. As the newer counting chambers are made of one piece of glass, the avoidance of cement solvents in cleaning the chamber is no longer necessary. Alcohol or ether must be used at times to remove grease. When cleansing the surface of the counting chamber and its cover with alcohol and ether, hold at the sides and below, or hold with metal tongs, to avoid getting natural oil from the fingers onto the glass surface.
- (2) *Pipette.*
- (a) Draw water through the pipette by suction (mouth suction, water pump, or air pump).
 - (b) Draw alcohol through the pipette by suction, to remove the water.
 - (c) Draw ether (or acetone) through the pipette, to remove the alcohol; continue the suction of air for a few seconds to remove the ether.



The method of blood counting is thoroughly explained herein. The numbers 1, 2, 3, 4 and the sixteen surrounding squares of each, indicate the parts of the slide used in counting white cells. The letters A, B, C, D, E, and the areas between the double lines, indicate the areas used in counting red cells.

Figure 3. Improved Neubauer counting chamber.

- (d) The small bead in the bulb should then shake about freely, indicating a clean and dry pipette.
- (e) If pipettes become plugged through neglect, clean the capillary bore with a horsehair and let the pipette stand a few hours filled with potassium bichromate-sulfuric acid cleaning solution or dilute nitric acid, then clean as above. A perfectly clean pipette stem should fill easily with blood by capillarity.

c. PROCEDURE.

- (1) Draw blood exactly to the 0.5 mark on the red-cell pipette. Remove any excess on the outside of the tip by wiping on a piece of gauze.

- (2) Draw up the diluting fluid exactly to the mark 101, making a dilution of 1:200.
- (3) Prevent loss of fluid from the pipette by holding the capillary point on the ball of thumb and kink the rubber tube at the end of the pipette, holding it against the middle finger. Shake in a figure 8 motion for 2 minutes to insure good mixing.
- (4) Put the cover glass in place on the counting chamber.
- (5) Discard 3 or more drops from the pipette, touch the tip of the pipette to the edge of the platform, and allow a thin layer of fluid to flow under the cover glass. If the fluid flows into the troughs, or there are bubbles under the cover glass, clean the counting chamber and try again.
- (6) Allow the cells to settle for 2 minutes.
- (7) Examine the chamber under the low-power (16-mm) lens of the microscope and note whether the distribution of red cells is even. If there is any clumping or grossly uneven distribution of cells, clean the counting chamber, shake the pipette again, and mount a new preparation.

d. COUNTING. Count all the cells in squares A, B, C, D, and E, inclosed by triple lines. (See fig. 3.) In counting cells in each square count all cells touching any of the triple lines on the right and top of the square. Do *not* count any cells touching the triple lines on the left and bottom of the square. From this count calculation is made of the number of cells per cubic millimeter of blood. A grossly uneven distribution of cells in the chamber usually results from faults in preparation (see below); when observed, the preparation and count should be repeated. Some variation in the counts of cells in different blocks of cells is, however, to be expected, as indicated by the example cited below, and further discussed below.

e. CALCULATION EXAMPLE.

(1) *Long method.*

- (a) Squares A, B, C, D, and E give counts of 100, 84, 90, 124, and 102 respectively, totaling 500 cells.
- (b) Therefore, 80 small squares, which occupy 80/400 or 1/5 sq mm, contain 500 cells.
- (c) Hence, 1 sq mm contains $5 \times 500 = 2,500$ cells.
- (d) Since this cell layer is 0.1 mm thick, 1 cu mm contains $10 \times 2,500 = 25,000$ cells.
- (e) Since the blood was diluted 1:200 it contains $200 \times 25,000 = 5,000,000$ cells per cubic millimeter.

- (f) *Summary.* Count of 80 small squares $\times$ 5 (for area) $\times$ 10 (for volume) $\times$ 200 (for dilution) equals number of cells per cubic millimeter of blood.
- (2) *Short method.* If the dilution was 1:200, the total cells per cubic millimeter may be found by adding four zeros to the total red-cell count in squares A, B, C, D, and E. For example, 500 with four zeros added is 5,000,000.
- f. **NORMAL RED-CELL (ERYTHROCYTE) COUNTS.** The normal count for men ranges from 4,600,000 to 6,200,000, and that for women from 4,200,000 to 5,400,000.

g. **SOURCES OF ERROR.**

- (1) Failure to hit the 0.5 mark exactly with the blood.
- (2) Allowing the blood to clot in the pipette.
- (3) Inaccurate dilution — either a bubble in the pipette or failure to exactly hit the 101 mark with the diluting fluid.
- (4) Failure to wipe away blood on the outside of the pipette.
- (5) Contamination of the diluting fluid with blood from red-cell pipettes.
- (6) Overfilling the chamber.
- (7) Not shaking the pipette long enough or allowing the cells to settle in the pipette after the shaking is stopped.
- (8) Dirty chamber or pipette.
- (9) Poor distribution in the chamber due to rouleaux formation or clumping.
- (10) Insufficient time allowed for the red cells to settle.
- (11) Yeast cells growing in the diluting fluid.
- (12) A poor sample of blood either by failure to mix venous blood in the bottle thoroughly or by obtaining blood diluted with tissue juice from the finger.
- (13) A visual error in counting the cells (an unexpected but frequent source of error, resulting especially in too low counts).

h. **ACCURACY OF COUNT.**

- (1) When the technics of sampling, sub-sampling, diluting, filling the chamber, etc., are effectively perfect, there remains a substantial error in the estimate of the total number of cells in the sample due to random settling of the cells on the squares of the chamber. This is the largest unavoidable source of error in the estimate. In careful experiments it has been found that the cells settle to give a distribution of varying numbers of cells in each square such that the error in the estimate of the total number of cells is equal to the square root of the

number of cells counted. Thus the expected percentile error in the estimate is less the greater the number of cells counted.

- (2) In routine practice, when only five large squares from a single specimen are counted and when technic is seldom perfect, it has been estimated that the error is ± 8 percent for a count of 5,000,000. If the usual practice of taking twice the error as effective limits is followed, the count may be expected to be determined significantly within ± 16 percent; that is, if the count is repeated many times with the same technique, the estimate 95 times out of 100 will be between 4,200,000 and 5,800,000 and 5 times out of 100 the count may fall beyond those extremes; in 50 times out of 100 the estimate will lie between 4,700,000 and 5,300,000. If greater accuracy than this is desired, two or more pipettes must be used and more squares must be counted.

63. White-cell (Leucocyte) Counts

a. MATERIALS.

- (1) The required materials are the same as those for a red-cell count except for the pipette and the diluting fluid.
- (2) The white-cell pipette (fig. 4) is similar to the red-cell

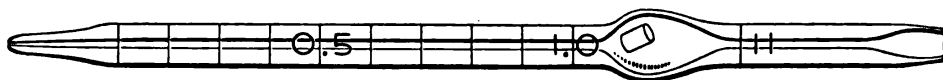


Figure 4. White-cell pipette

pipette, but has a smaller bulb, which contains a small white bead, and produces less dilution of the blood. The fifth line on the graduated capillary tube is marked "0.5," the tenth line "1.0," and above the bulb "11."

- (3) The diluting fluid is prepared as follows:

Glacial acetic acid	-----	3 ml
Distilled water	-----	97 ml

This fluid may be tinted blue, for convenience in identifying it, by addition of a drop of 1 percent gentian violet.

b. PROCEDURE.

- (1) Draw blood to the 0.5 mark.
- (2) Draw diluting fluid to the 11 mark, making a dilution of 1:20.
- (3) Shake, as in red-cell counting.

- (4) Discard 3 or 4 drops, and then fill the counting chamber.
- (5) Allow the cells to settle.
- (6) Examine under the low power of the microscope.

c. COUNTING. The white cells are counted in the four large corner squares labeled 1, 2, 3, and 4. (See fig. 3.) The difference between the largest and smallest number of cells in any two squares should not exceed 10.

d. CALCULATION EXAMPLE.

(1) *Long method.*

- (a) Squares 1, 2, 3, and 4 give counts of 34, 42, 38, and 36 respectively, totaling 150 cells.
- (b) Hence, one square (1 sq mm) contains $150 \div 4 = 37.5$ cells.
- (c) Since the cell layer is 0.1 mm thick, 1 cu mm contains $37.5 \times 10 = 375$ cells.
- (d) Since the blood was diluted 1:20, it contains $20 \times 375 = 7,500$ cells per cubic millimeter.
- (e) *Summary.* Count of four squares: 4 (for average single square) $\times 10$ (for volume) $\times 20$ (for dilution) equals number of cells per cubic millimeter of blood.

(2) *Short method.* Multiply the number of cells in four large squares (0.4 cu mm) by 50. For example, $150 \times 50 = 7,500$.

e. NORMAL WHITE-CELL (LEUCOCYTE) COUNT. Normally, this count is 5,000 to 10,000. Many normal persons have variable counts according to activity, time of day, etc. Daily counts on a patient should be done at the same time every day. In cases in which the white count is very high, it may be necessary to use a dilution of 1:100, using the red-cell pipette and changing the calculation accordingly. In cases in which the count is abnormally low, make the dilution 1:10 by drawing blood in the white-cell pipette to the 1.0 mark, and multiply the number of cells counted in four large squares by 25.

64. Cell Count Cerebrospinal Fluid

a. The total cell count in cerebrospinal fluid should be made at once before the fluid has formed a coagulum and before cells have deteriorated. Coagulation of the fluid may be prevented by placing a minute amount of potassium oxalate in the tube to be used for the cell count. The presence of red blood cells due to bleeding caused by the puncture invalidates the count and may be avoided by collecting the specimen in two or three tubes and performing the count on the second or third tube which will usually be free of blood.

b. Prepare diluting fluid as follows:

Crystal violet -----	0.2 gm
Glacial acetic acid -----	10.0 ml
Water, distilled -----	90.0 ml
Filter	

c. Shake the spinal fluid to secure an even suspension of cells. Draw diluting fluid to the mark 1. in a white cell counting pipette and draw spinal fluid to the mark 11. Shake the pipette, discard 2 or 3 drops and fill the counting chamber of a hemocytometer.

d. Count all cells (erythrocytes are laked by the diluting fluid) in the entire ruled area, 9 large squares, which is 0.9 cu mm.

11

Multiply by $\frac{11}{9}$ to obtain the number of cells per cubic millimeter of spinal fluid.

e. The Fuchs-Rosenthal counting chamber is designed specially for spinal fluid and is larger than the blood counting chamber. The ruled area covers 16 sq mm and depth of the chamber is 0.2 mm. All cells in the ruled area are counted which gives the number of

11

cells in 3.2 cu mm. Divide this figure by 3.2 and multiply by $\frac{11}{10}$,

the dilution factor, to obtain the number of cells per cubic millimeter. In practice it is sufficiently accurate to divide the number of cells in the ruled area by 3.

f. With the high dry power of the microscope, polymorphonuclear leucocytes can usually be distinguished from lymphocytes and a differential count can be made, if the cells are not too numerous. A more accurate differential count can be made by preparing a thin smear from the centrifugalized spinal fluid sediment. It may be necessary to use egg albumin or a cellular serum to make the spinal fluid adhere to the slide. Stain the smear with Wright's stain in the same manner as a blood smear and count 100 cells.

g. In adults, the normal cell count is 0 to 10 cells but in children under 1 year of age, the count may be as high as 30 cells and from 1 to 5 years, 20 cells may be found. Normally all cells are lymphocytes.

65. Blood Films

a. CLEANING OF NEW SLIDES AND COVER GLASSES.

(1) A prerequisite in making a good blood film is to have chemically clean slides and cover glasses. First wash in soapy water and rinse thoroughly with water.

- (2) Place slides in a large beaker of 95 percent ethyl alcohol.
- (3) Dry and polish with a soft lint-free cloth (not gauze).
- (4) Flame over a Bunsen burner.
- (5) Place in box with a clean slip of paper between each pair of slides.

b. DIRTY SLIDES.

- (1) Boil in a 5 percent aqueous solution of sodium bicarbonate.
- (2) Scrub with soap and water.
- (3) Place in cleaning solution (potassium bichromate-sulfuric acid) for 12 hours.
- (4) Wash thoroughly to remove all traces of acid and place in 95 percent ethyl alcohol as for new slides.
- (5) Discard all slides that are badly scratched or discolored.

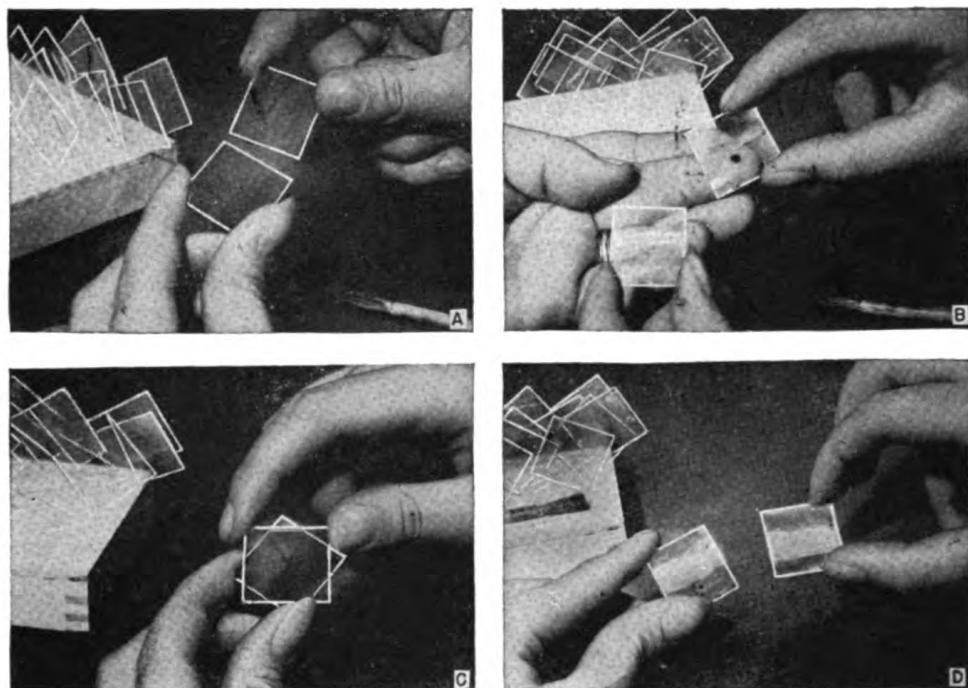
c. COVER GLASSES. These are cleaned in the same way as new slides except that they should not be flamed. Careful wiping without pressure will prevent much breakage.

d. PREPARATION OF BLOOD FILMS.

- (1) *Materials.* The following materials are required: Equipment for finger puncture and clean cover glasses and slides, free from grease.
- (2) *Preliminary details.* Use thoroughly cleaned glassware. No. 2 cover glasses, 22 mm square are preferable. Slides or cover glasses must be flat. Avoid concave or convex surfaces, since the blood will not spread properly. Wipe off the first drop of blood from the finger and use the second, which is more representative of the circulating blood.

e. SMEARING.

- (1) *Cover glass method* (fig. 5).
 - (a) Pick up two cover glasses, touching only the edges.
 - (b) Adjust these between the thumb and forefinger of the left hand. This leaves the right hand free to regulate the size of the drop of blood to be picked up.
 - (c) Hold the bottom cover glass of the two held in the left hand just above the drop of blood coming from the patient's finger. Let the blood well up to reach the cover glass. Never touch the skin with the cover glass. The drop of blood should be about the size of a small black-headed pin.
 - (d) With the right hand remove the other cover slip from its position in the left hand and place the two together



- A. Holding both cover slips.
- B. Touching drop of blood.
- C. Approximating both cover slips.
- D. Drawing cover slips apart.

Figure 5. Method of making cover slip smears.

in such a manner that the drop of blood spreads without causing bubbles.

- (e) Let the blood spread until it has *almost* stopped spreading.
 - (f) Pull the cover glasses apart, doing so in the absolutely horizontal axis. Any tendency to a vertical pull ruins the preparation, since holes in the smear result.
 - (g) Place the preparations (smeared side up) on clean paper and allow them to dry in the air.
- (2) *Slide method*
- (a) Place a small drop of blood on the end of a slide and lay the slide on a flat surface.
 - (b) Hold a second slide between the thumb and third finger of the right hand, and place one end at a 30° angle on the slide with the drop of blood.
 - (c) Pull back the upper slide until it touches the drop of blood which then spreads along from one side to the other of the end of the top slide. (See fig. 6.)

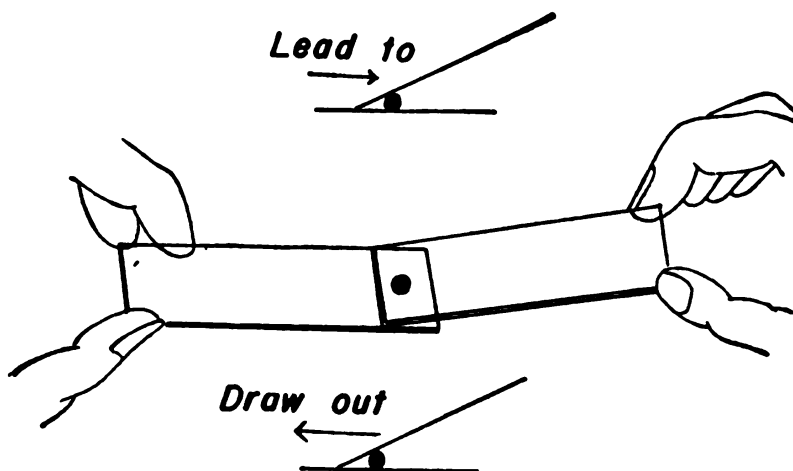


Figure 6. Method of making blood film.

- (d) Holding the lower slide with the left hand, push the upper slide with a firm, steady motion toward the opposite end of the lower slide. The slower the movement, the thicker the film; the greater the angle, the thicker the film. *Thin smears are necessary for satisfactory differential counts.* Avoid all unnecessary pressure because of the fragility of the cells.
- (e) Allow the slide to air-dry. In areas where insects are abundant, slides must be protected or they will be ruined.
- (f) Labeling may be done by writing on the thicker end of the film with an ordinary lead pencil when the film is dry. The slides should be stained within 24 hours for best results.

f. CRITERIA FOR GOOD SMEARS.

- (1) *Cover glass method.* Smooth, even spreads with red cells lying flat (never in rouleaux). Their edges may touch, but there must be no overlapping. At least eight such areas without holes and without streaks, as observed with the low-power lens of the microscope, constitute the minimal requirements for a good smear.
- (2) *Slide method.* A good film should be smooth and without waves. The edges should be even, and the film should not extend to the edges or end of the slide.

g. RELATIVE MERITS OF COVER GLASS AND SLIDE METHODS.

- (1) The cover glass method gives a much better distribution of white-cells and is the method of choice in making differential white-cell counts.

- (2) The slide method gives a good distribution of red cells. The large white cells (polymorphonuclears and monocytes) are pushed to the edges, and the lymphocytes remain scattered through the spread, which makes satisfactory differential counting impossible. When red cells are to be examined for parasites, the slide method is superior to the cover glass method, inasmuch as more fields are available for study and red-cell distribution is as good as with the cover glass method. (See also par. 463 for malaria blood films.)

66. Staining of Blood Films

a. REAGENTS.

- (1) *Wright's stain*. This stain is used for routine blood films and for other laboratory purposes. The powdered stain is issued from the supply table. The stain solution is prepared by the laboratory as follows:

Wright's stain powder	0.3 gm
Glycerol (neutral, chemically pure)	3.0 ml
Methyl alcohol (absolute and acetone-free) ..	97.0 ml

Put the powder in a dry mortar; grind with a pestle; add the glycerol and grind together thoroughly. Add the methyl alcohol and mix. Allow to stand overnight in a tightly stoppered flask; then filter, and set aside for a few days before use. Age improves the stain. The glycerol may be omitted when air humidity is high.

- (2) *Buffer solution*. This is prepared as follows:

Potassium acid phosphate	1.63 gm
Disodium phosphate	3.20 gm
Distilled water	1,000 ml

b. PROCEDURE.

- (1) Cover the dried film completely with Wright's stain for 1 minute. This fixes the blood film. Corks are convenient for holding cover glasses while staining.
- (2) Add the buffer solution to the stain, drop by drop, until a greenish, metallic scum appears on the surface. The stain and buffer should cover the cover glass or slide, but none should run off. Determine the time for staining by trial with a series of slides. This is usually about 2 to 5 minutes, but is variable with every batch of stain. The color of the cells may be varied by changing the pH of the buffer solution. The granules in the neutrophils should stain lilac, the eosinophils bright red, and the basophils deep blue.

- (3) Wash with water, continuing until the film is lavender pink. In washing, float off, rather than wash off, the scum with a stream of water (preferably distilled), which is at first run very slowly, and then more briskly, to free the smear from all traces of excess stain. All this time the cover glass on slide must remain absolutely horizontal. Washing should take from 5 to 10 seconds.
- (4) After washing is completed, the cover glass or slide is tilted and the lower edge touched to a blotter so that the excess of water is quickly drained off.
- (5) The smears are then dried in one of the following ways:
 - (a) The smear may be air-dried by placing the cover glass or slide against some support and letting one edge rest on a blotter, or it may be waved gently in the air, touching only the edge of the cover glass or slide.
 - (b) The cover glass or slide may be placed between layers of fine blotting paper that is absolutely free from dust. Apply light pressure to the blotting paper to facilitate drying. Then pick up the preparation and move it to a dry place, again applying light pressure. Be careful not to push the smear along the blotter or to press it too heavily, for holes and streaks in the preparation will be the result. The staining is thought to be better if the blotting method is employed, but many good smears have been ruined by slight negligence in the manner of blotting.
- (6) After drying the preparation, cover glasses should be mounted in clarite or in gum damar. (To prepare gum damar for use as a mounting fluid, make a dilute solution of the gum in xylol; add sufficient calcium carbonate to make the mixture appear smoky, and allow this to season by letting it stand for 2 to 3 months.) If the slides are used, the preparation usually is not mounted, and for examination, immersion oil is applied directly to the surface of the smear. If desired, a large, thin cover glass, mounted in clarite or gum damar, may be used to guard the smear on the slide.

c. COMMON PITFALLS IN STAINING.

- (1) The stain precipitates on the preparation. This is due to faulty washing, that is, not holding the preparation horizontally and floating off the scum, thus permitting the latter to touch the smear, or dust to settle on the smear. It can be prevented by holding the preparation horizontally during the washing and learning to play the stream of water to the best advantage in washing and

by keeping the smears clean during the interval that elapses between pulling and staining.

- (2) An indefinite scum is present between the cells, due to insufficient washing.
- (3) If, in spite of these precautions, there is still some precipitate on the preparations, and the general color is too blue, this can usually be avoided by diluting the stain with an equal part of absolute methyl alcohol and applying the diluted stain as directed above. A poor preparation may sometimes be saved by washing very rapidly with a dash of 95 percent alcohol and again washing immediately in water. If this is attempted, the alcohol should not be allowed to remain in contact with the smear for more than 1 second.
- (4) The preparations are damaged by improper blotting.
- (5) The Wright's stain may deteriorate owing to volatile acids kept in the same place, to the addition of water from the mixing pipettes, or to an unclean storage bottle.

d. **CRITERIA FOR WELL-STAINED PREPARATIONS.** As seen with the naked eye, the blood film covers about three-quarters of the cover slip and the film is yellowish or pink. All traces of blue should have been washed away. As seen microscopically, the red cells should be buff, neither lemon nor red. The platelets should be well stained and purple-blue, with their architecture plainly visible. The white cells should be stained as described in the section covering their characteristics. The areas between the cells should be clear and free from all suggestions of stain. The cells should stand out with distinctness, with no suggestion of hazy edges. There should be no precipitate.

e. **ALTERNATIVE METHOD FOR PREPARATION OF WRIGHT'S STAIN.**

Wright's stain powder	1.5 gm
Potassium acid phosphate	0.4 gm
Disodium phosphate	0.6 gm
Methyl alcohol (absolute and acetone free)	500 ml

Grind the dry ingredients in a mortar, and then add the alcohol. Allow the stain to stand for several days, shaking occasionally during this interval. Before use, filter.

When the stain has been prepared with buffer salts, distilled water is added to the stain in *b* (2) above, instead of buffer solution.

One or 2 drops of weak acetic acid solution or of weak ammonia water serve as simple acid and alkaline solutions when they are needed to correct stains that are too alkaline or acid, respectively.

67. Differential White-cell Counts

a. With a mechanical stage count across and up and down the good areas of the spread. Be careful to use such a technic that the same areas will not be counted twice.

b. One hundred white cells are usually counted, but the number may be varied according to the number of leucocytes present. When there is leucopenia, fewer cells may be examined, whereas when there is leucocytosis, more cells should be studied. In order to get the true proportion, it is necessary to include unclassified cells as well as those that can be classified.

c. A white cell count should always be made at the time a differential count is done. It is only by this means that absolute changes in the number of cells can be determined.

d. The normal limits of relative and absolute white-cell counts are listed in table I.

Table I. Relative and Absolute Normal White-Cell Counts

Type of cell	Percent	Absolute number		
		Average	Minimum	Maximum
Total leucocytes -----	-----	7,000	5,000	10,000
Myelocytes -----	0	0	0	0
Unsegmented neutrophils -----	4-6	400	200	600
Segmented neutrophils -----	56-62	4,200	2,800	5,800
Eosinophils -----	1-3	200	200	300
Basophils -----	0-0.75	35	15	75
Lymphocytes -----	20-30	2,000	1,000	3,000
Monocytes -----	4-8	450	300	600

68. Characteristics of Stained Cells

a. RED CELLS. Normal red cells (erythrocytes) are circular, non-granular, non-nucleated cells, the centers of which are less intensely colored than the borders. In various diseases the blood may contain red cells showing the following abnormalities:

- (1) *Hypochromia*. The cells are pale staining due to a decreased amount of hemoglobin.
- (2) *Polychromasia*. The cells are bluish rather than buff.
- (3) *Anisocytosis*. There is a wide variation in the size of the cells.
- (4) *Poikilocytosis*. Many of the cells are not circular. The abnormal forms include oval-shaped cells, those looking like cigars, sickles, pears, bottles, and bells, and those resembling doughnuts and targets.
- (5) *Macrocytosis*. The average size of the cells is greater than the normal (7.5 microns).

- (6) *Microcytosis*. The average size of the cells is smaller than the normal.
 - (7) *Stippling*. The cells contain a fine or coarse dusting of bluish-black granules (seen in lead poisoning and other conditions).
 - (8) *Howell-Jolly bodies*. The cells contain one or two small, blue-black dots.
 - (9) *Reticulocytes*. These cells have bright blue, irregular threads and granules within the cell. Unless the preparation has been stained first with brilliant cresyl blue (par. 75), reticulocytes will not be seen in Wright-stained smears.
 - (10) *Nucleated red cells*. These may resemble the red cells except for the presence of a nucleus (orthochromatic normoblast), or their cytoplasm may be bluish (polychromatic normoblast) or deep blue (basophilic normoblast). In pernicious anemia and related conditions various types of more primitive forms (megaloblasts) may appear.
 - (11) *Parasites*. These may be contained within the red cells (malaria, bartonella, etc.).
- b. WHITE BLOOD CELLS (LEUCOCYTES).

(1) The classification of cells found in normal blood (figs. 7 and 8 ② and table 2) is as follows:

- (a) *Metamyelocyte or juvenile*. A metamyelocyte is a cell of the granulocytic series having specific granules in the cytoplasm and a nucleus intermediate in shape between that of a myelocyte and the band cell. The nucleus usually has an indented oval shape, resembling a bean or kidney.
- (b) *Band cell*. A band cell is a cell of the granulocytic series which has a nucleus that could be described as a curved or coiled band, no matter how marked the indentation, if it does not completely segment the nucleus into lobes connected by a filament. It is differentiated from the metamyelocyte by an appreciable length of the nucleus having parallel sides, and from the segmented neutrophil by having no indentation which could be described as a filament.
- (c) *Polymorphonuclear neutrophils (P.M.N.)*. The nuclei are irregular, having two, three, or more lobes and often appearing polynuclear. The deep purple, reticular, intranuclear, chromatin is pronounced. The cytoplasmic granules are pink or violet.

Table II. Morphologic Characteristics of the Leucocytes (Wright's Stain)

Type of cell	Size	Nucleus						Cytoplasm			
		Position	Shape	Color	Chromatin	Nuclear membrane	Nucleoli	Relative amount	Color	Perinuclear clear zone	Granules
1. Granulocytes:											
(a) Myeloblast.....	10-18 μ	Eccentric or central.	Round or oval.	Light reddish-purple.	Very fine meshwork.	Very fine.....	2-5.....	Scanty...	Deep blue.	None...	None, or only a few (azurophilic or myeloid).
(b) Myelocyte.....	12-18 μ	Eccentric.....	Oval or slightly indented.	Reddish-purple.	Fine but becomes gradually coarser.	Indistinct.....	Smaller, fewer.	Moderate.	Bluish pink.	None...	Red or blue-black, fine or coarse..
(c) Metamyelocyte and band cell	10-18 μ	Central or eccentric.	Horseshoe or sausage.	Light purplish-blue.	Base and oxychromatin clearly distinguished.	Present.....	None.....	Plentiful...	Pink...	None...	Neutrophilic, eosinophilic, or basophilic.
(d) Polymorphonuclear neutrophil P.M.N.	10-15 μ	Central or eccentric.	2-5 or more lobes.	Deep purplish-blue.	Rather coarse.	Present.....	None.....	Plentiful...	Faint pink.	None...	Fine pink or violet-pink.
(e) Polymorphonuclear eosinophil P.M.E.	10-15 μ	Central or eccentric.	2-3 lobes.	Paler purplish-blue.	Coarse.....	Present.....	None.....	Plentiful...	Bluish-pink.	None...	Large, coarse, uniform in size, crimson-red, numerous.
(f) Polymorphonuclear basophil P.M.B.	10-15 μ	Central.....	2-3 lobes.	Pale purplish-blue.	Coarse, overlaid with granules.	Present.....	None.....	Plentiful...	Faint pink.	None...	Large, coarse, uniform, bluish-black.
2. Lymphocytes											
(a) Lymphoblast.....	10-18 μ	Eccentric or central.	Round or oval.	Light reddish-purple.	Moderately coarse particles "stippled."	Fairly dense.	1-2.....	Scanty...	Deep blue.	Present...	None.

(b) "Mature" lymphocyte.	7-18 μ	Eccentric----	Round or slightly indented.	Deep purplish- blue.	Large masses of moderate or large size, or pyknotic.	Dense-----	None-----	Scanty or plentiful.	Sky blue, deep blue, or even pale pink.	Present if cyto- plasm is dark	None or few azurophilic.
3. Monocyte-----	12-20 μ	Eccentric or central.	Oval, round, notched, or horseshoe.	Pale bluish- violet.	Fine reticulated, skeinlike, or lacy.	Present-----	None-----	Abundant	Grayish or cloudy blue.	None----	Abundant, fine lilac or reddish blue.
Clasmatocyte-----	15-80 μ	Central-----	Elongated, indented, or oval.	Pale bluish- violet.	Spongy-----	Distinct----	None-----	Usually abun- dant.	Opaque sky blue.	None----	Numerous, moderately coarse azur granules and vacuoles.

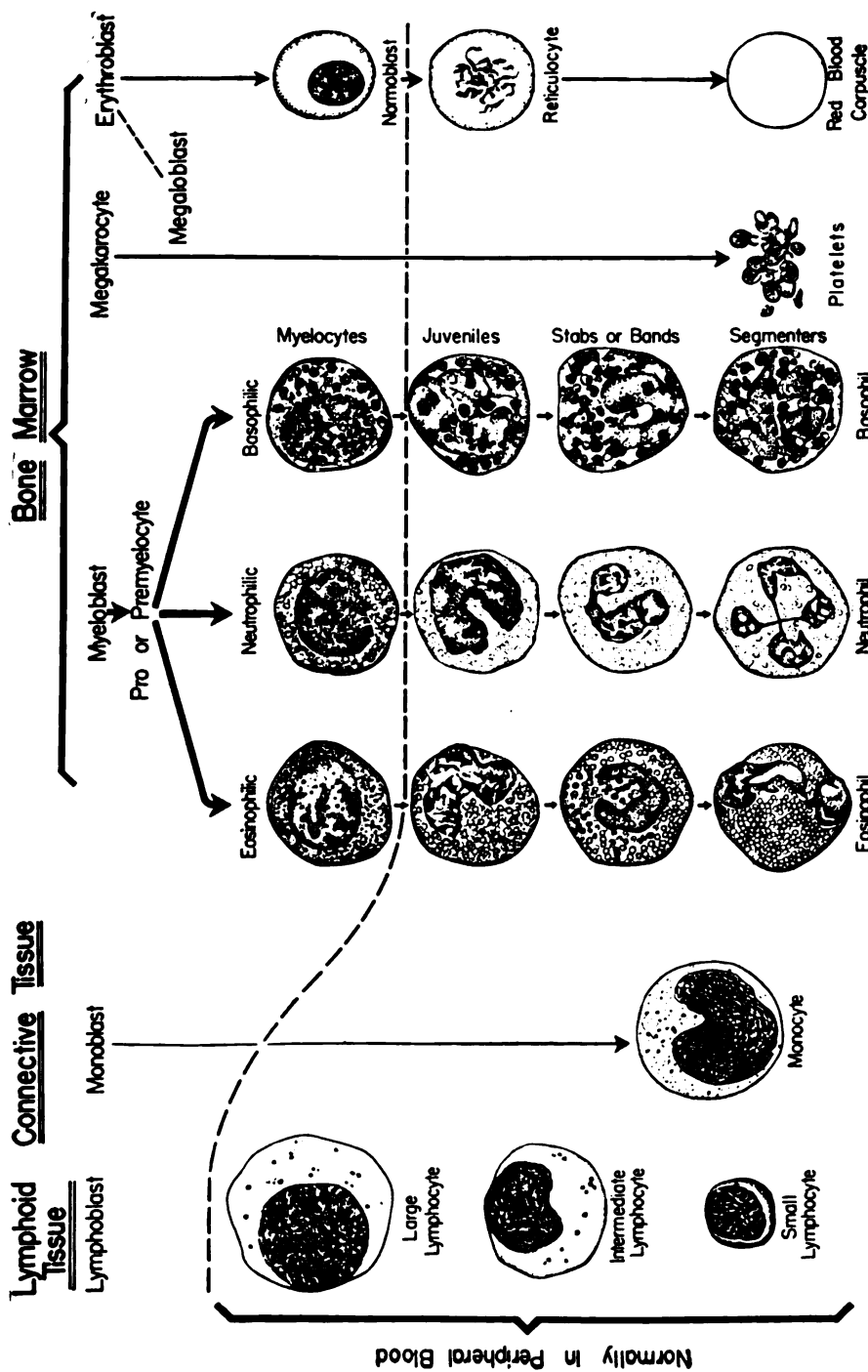
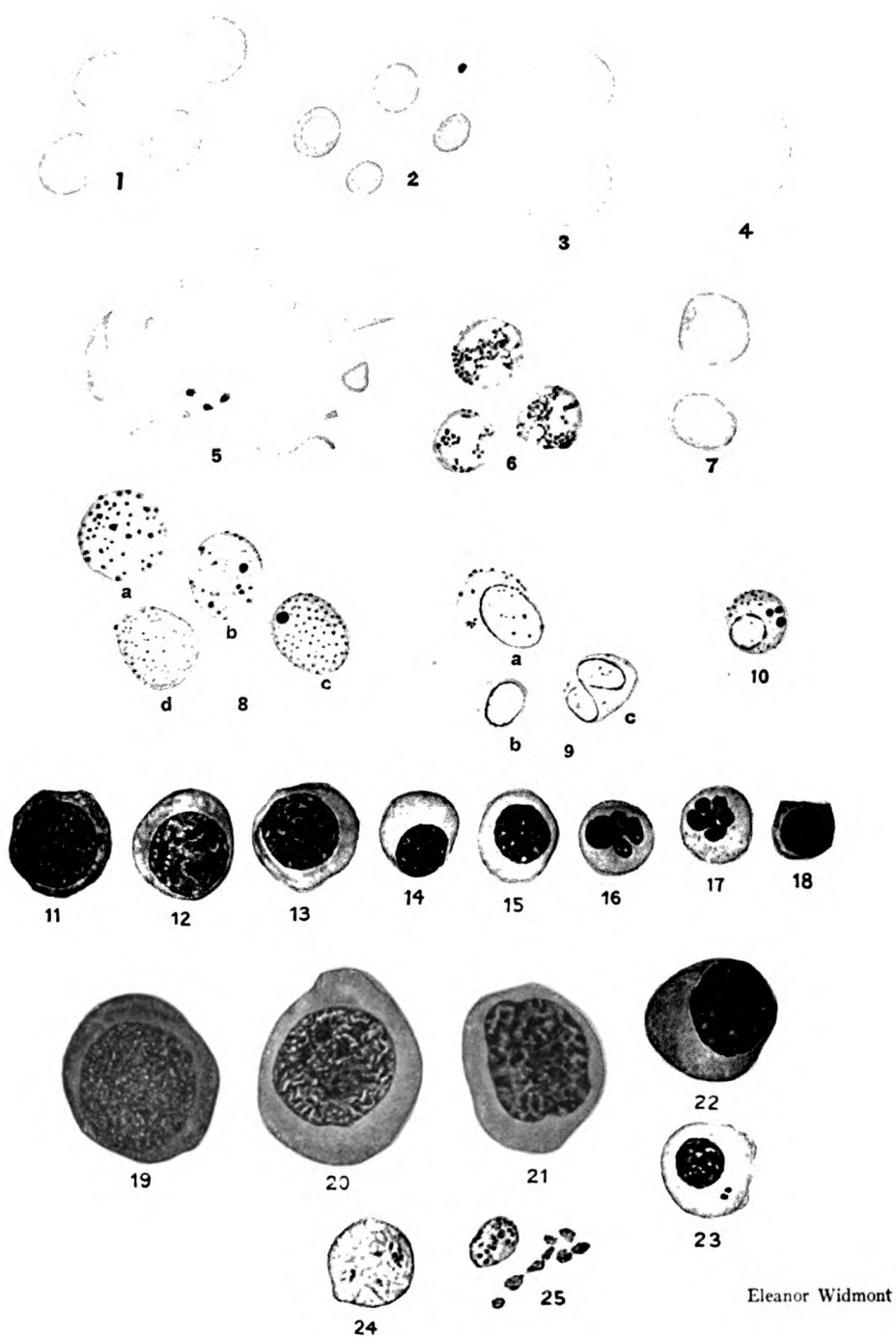


Figure 7. Schematic Representation of the Origin and Development of Blood Cells.

- (d) *Polymorphonuclear eosinophils (P.M.E.)*. The nucleus is irregular, generally bilobed, but may give the appearance of being actually polynuclear. The cytoplasmic granules are larger when compared with the neutrophilic granules, and are all of the same size. In poorly stained films the granules may be paler, being nearly the same color as the neutrophilic granules; less often they take a dirty brownish stain. In general, eosinophils are recognized by the large, uniform size of the granules rather than by the depth of the granular stain. These cells tend to be a trifle larger than the neutrophils and are rather fragile. For the latter reason they can often be found broken, with their granules scattered.
 - (e) *Polymorphonuclear basophils (P.M.B.)*. The nucleus is of the size and shape of the eosinophilic nucleus, but sometimes is entirely hidden by the deeply staining basophilic (blue) granules that completely fill the cells.
 - (f) *Lymphocytes*. The cell is fairly regular in outline, and round in shape. The nucleus is round or slightly notched, and takes a deep blue stain. The chromatin masses are coarse. The protoplasm stains deep to pale blue. In about one-third of these cells, so-called "azure granules" can be seen; there are usually few in number and variable in size and are stained purplish-red.
 - (g) *Monocytes*. These are large cells with a single nucleus that is round or indented to a variable degree. The nuclear stain is lighter than that of the lymphocyte, and the nucleus appears less dense. The chromatin network is fine, but a few denser aggregations of chromatin are frequently found. In the cytoplasm, which is stained cloudy blue or gray, are abundant fine, lilac or reddish-blue granules.
- (2) Under abnormal conditions, other types of leucocytes are found in the blood. These include the following:
- (a) *Immature forms*. In certain diseases, younger forms of the granulocytic, lymphocytic and monocytic series of cells are present. (See figs. 7 and 8 and table II.)
 - (b) *Toxic neutrophils*. Toxic neutrophils are cells which show one or more qualitative changes such as the presence of relatively large, deeply staining cytoplasmic granules, basophilia of the cytoplasm, vacuoles, and condensation of nuclear chromatin. Usually the more "toxic" the reaction, the fewer the granules and the

1. Normal red corpuscles (normocytes).
2. Small red corpuscles (microcytes).
3. Large red corpuscles (macrocytes).
4. Exceptionally large red corpuscle from a case of pernicious anemia.
5. Abnormally shaped red corpuscles (poikilocytes) from cases of pernicious anemia, chronic posthemorrhagic anemia, and sickle cell anemia.
6. Reticulocytes, stained with cresyl blue, as well as Wright's solution, to show the granulo-reticulofilamentous network.
7. Red corpuscles showing polychromatophilia (diffuse basophilia).
8. Red corpuscles showing basophilic stippling (Punctate basophilia): *a*, *b*, and *c* are from a case of pernicious anemia, *d* from a case of lead poisoning. The stippling in *d* is much finer than in the other cells; *c* contains a large nuclear fragment; *b* and *d* are also diffusely basophilic.
9. Red corpuscles containing Cabot ring bodies. The cytoplasm of *a* and *c* is diffusely basophilic and contains fine "chromatin dust."
10. Basophilic red corpuscle containing 3 Howell-Jolly bodies, a Cabot ring body, and fine "chromatin dust."
- 11 to 18. Normoblasts. Numbers 11 to 17 from the bone marrow of cases of hemolytic anemia, 18 from a case of hypochromic microcytic anemia.
11. Pronormoblast. There are a few tiny nucleoli in the nucleus. The cytoplasm is deeply basophilic, but hemoglobin formation is just commencing at the four o'clock position.
12. Basophilic normoblast. There are no nucleoli, the cytoplasm is less deeply basophilic than that of cell 11. There is beginning hemoglobin formation in the upper portion of the cell.
13. Polychromatic normoblast.
- 14 to 17. Orthochromatic normoblasts. In Nos. 16 and 17 there is karyorrhexis. In No. 17 there is also punctate basophilia.
18. Basophilic "microblast," to be distinguished from a lymphocyte by the opaque grayish-blue color of the cytoplasm and the small size of the cell.
- 19 to 23. Megaloblasts from the bone marrow of cases of pernicious anemia.
19. Promegaloblast. Note that the chromatin appears finely granular and is much more homogeneous than that of the pronormoblast (N. 11). The nucleoli are barely perceptible.
20. Basophilic megaloblast. Note the finely divided, meshlike chromatin, so characteristic of the megaloblast. Compare with cell 12.
- 21 and 22. Polychromatic megaloblasts. Not only is the color of the cytoplasm changing as compared with that of cells 19 and 20 but the chromatin is becoming aggregated into larger masses. It continues to be rather homogeneous.
23. Orthochromatic megaloblast containing 2 Howell-Jolly bodies.
24. Giant platelet.
25. A few platelets of normal size and one exceptionally large one.

Figure 8. ①



Eleanor Widmont

Figure 8. ① Normal and abnormal red corpuscles and platelets.
(Wright's stain. 1 mm = 1 μ .)

larger their size. Obviously the effects of poor staining must be distinguished from the changes described above.

- (c) In differentiating leucocytes it is important that all the detailed characteristics recorded in table II be observed. It should become a habit to note, in examining a cell, the size, shape, position, and chromatin structure of the nucleus, the presence or absence of nucleoli, the relative amount and color of the cytoplasm, the presence of a perinuclear clear zone, and the presence and nature of cytoplasmic granules. *One should be careful, however, to identify a cell by all of these features and not to be guided by one cell characteristic alone.* A well-made, well-stained blood smear is an absolutely essential prerequisite for the identification of leucocytes.

69. Fresh (Wet) Blood Preparations

a. PURPOSES. The examination of a wet, unstained film of blood is useful for several reasons:

- (1) No stain is required.
- (2) Parasites, if present, are not killed and they may be picked up easily by their movement in the preparation (trypanosomes, spirochetes of relapsing fever, and microfilaria) or by their pigment (malaria).
- (3) Sickling of red corpuscles can be observed.
- (4) The variations in size and shape of the leucocytes and their motion can be observed.

b. TECHNIC.

- (1) Place a drop of blood the size of a pinhead on a cover glass.
- (2) Place the cover glass on a glass slide in such a manner that the drop is spread without bubble formation.
- (3) Ring the cover glass with vaseline to prevent drying.
- (4) Study first with the lower power, then with high dry and oil immersion.

c. CRITERIA FOR A GOOD PREPARATION. The spread must be thin, with the cells lying flat and not overlapping.

d. COMMON DIFFICULTIES IN MAKING A GOOD PREPARATION. The chief causes of poor preparation are dirty glassware and careless spreading of the drop.

- 1 to 13. *Granulocytic series*. Numbers 1 to 11 are arranged in order of maturity.
1. Myeloblast. The nuclear chromatin is very fine, there is only a faint nuclear membrane, and the nucleus contains 6 nucleoli. The cytoplasm is deeply basophilic and contains no granules.
 - 2 to 6. Myelocytes, in order of maturity. Number 2, which some writers would call a myeloblast and others a promyelocyte or myelocyte "A," differs from No. 1 only in that there are a few granules in the cytoplasm; Nos. 3 and 4 are also spoken of as promyelocytes, or as myelocytes "B" because their cytoplasm contains a moderate number of granules. In No. 4 a few granules are seen overlying the nucleus which is becoming less distinct than that of myeloblasts and very young myelocytes. Numbers 5 and 6 represent the typical "differentiated" neutrophilic myelocyte or myelocyte "C" — there are many granules in an abundant cytoplasm, and the nucleus is relatively indistinct and has the appearance of lying deep in the cell.
 7. Metamyelocytes, or "juveniles." The nucleus has again become distinct; the basichromatin is more compact than in the younger cells of this series.
 8. Band cell. An appreciable length of the nucleus has parallel sides.
 - 9 to 11. Polymorphonuclear neutrophils with 2, 3, and 9 segments, respectively. Number 11 represents the giant, multilobed neutrophil which is most commonly seen in pernicious anemia in relapse.
 12. Basophil. The granules are characteristically very large and bluish-black in color. The nucleus is not readily distinguished.
 13. Eosinophil. The granules are numerous, large, uniform in size, and brick-red in color.
 14. Portion of a megakaryocyte found in the blood.
 15. Degenerated nucleus, a so-called basket cell.
 - 16 to 21. *Lymphocytic series*.
 16. Lymphoblast. The nuclear chromatin is fine and somewhat stippled. There is a distinct membrane about the nucleus and around the 3 nucleoli, and there is more of a tendency to clump than in the myeloblast. The cytoplasm is deeply basophilic.
 - 17 to 21. Mature lymphocytes. The nuclear chromatin is more compact than that of the lymphoblast and there are no nucleoli. There is a perinuclear clear zone in some of the cells. The cytoplasm is quite basophilic in No. 17 but pale in the others. Azurophilic granules are seen in some of the cells. The light areas in the nucleus of No. 19 should not be confused with nucleoli.
 - 22 to 25. *Monocytes*. The nuclear chromatin is quite fine and strandlike and thus differs from that of the myeloid and lymphocytic series of cells. The cytoplasm contains many fine, lilac or reddish-blue granules.

Figure 8. ②

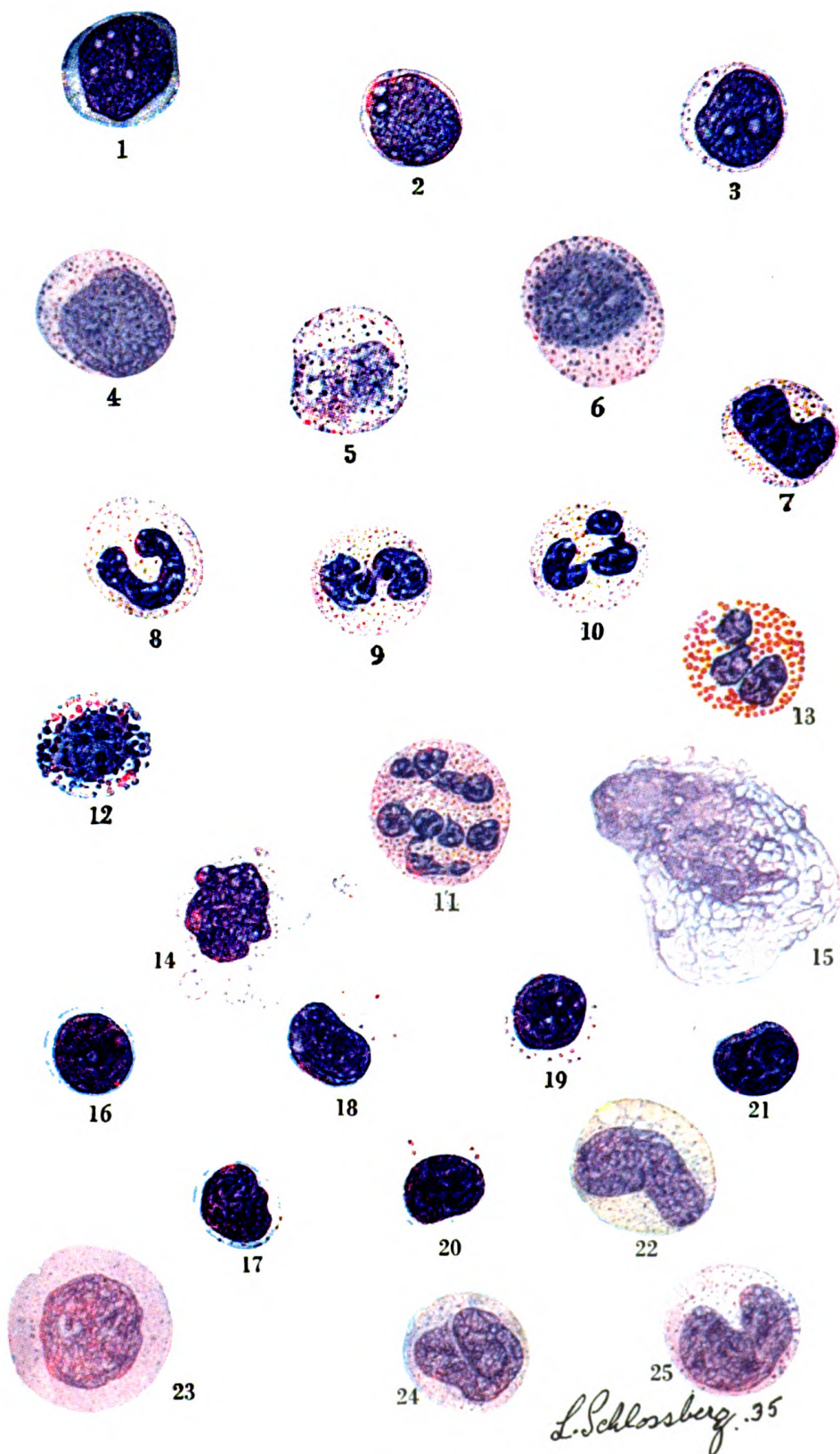


Figure 8. ③ Normal and abnormal white corpuscles.
(Wright's stain. 1 mm = 1 μ .)

70. Use of Venous Blood for Hematologic Procedures ("Screen Tests")

a. GENERAL. When venipuncture is possible or is being done (for routine serologic tests for syphilis or for blood chemistry), it is simpler to collect blood from a vein in a bottle containing the proper anticoagulant and to send it to the laboratory than to have a technician come to the patient with a tray of pipettes and diluting fluids and a hemoglobinometer. Furthermore, if venous blood is available, the following so-called "screen test" yields more extensive and more accurate information and requires less highly trained personnel than the usual routine of red-cell and white-cell counts and a hemoglobin determination:

- (1) Fill a hematocrit tube (par. 71) and allow it to stand vertically for 1 hour, thus determining the sedimentation (par. 74).
- (2) Centrifuge the hematocrit tube, as directed in paragraph 71 and read the volume of packed red cells and the volume of packed leucocytes and platelets. The volume of packed red corpuscles is an accurate index of the presence of anemia or polycythemia. The volume of packed leucocytes and platelets normally is 0.7 to 1.0 mm. If this layer is greater or less than this, an increase or reduction in leucocytes or platelets (or both) is suggested and appropriate counts should be made.
- (3) The color of the plasma is compared with a series of standards (par. 73) and the icteric index is determined.
- (4) If any of the above determinations reveal some abnormality, further studies should be made as indicated. Venous blood is satisfactory for the determination of hemoglobin, red-cell count, white-cell count, platelet count, reticulocyte count, and red-cell fragility. These can be *carried out without recourse to the patient for additional blood*. If any of these measurements require repetition or checking, blood is available for this purpose. Venous blood is *not* suitable for morphologic studies, and blood smears should always be made directly from the finger or ear lobe.

b. VENIPUNCTURE. The technic of venipuncture is given elsewhere. (See par. 50.) The needle and syringe must be *dry* so that no hemolysis will occur.

c. ANTICOAGULANT AND BOTTLE FOR COLLECTING VENOUS BLOOD.

- (1) The anticoagulant solution is prepared as follows:
Ammonium oxalate ----- 1.2 gm

Potassium oxalate -----	0.8 gm
Distilled water -----	100 ml

One-tenth of a milliliter of this solution (2 mg of oxalate) will prevent the coagulation of 1 ml of blood.

- (2) The most suitable bottle is one of somewhat more than 5 ml capacity with a mouth large enough to permit the entry of counting pipettes and supplied with a cork that allows no leaking of blood even when the bottle is inverted.
- (3) Drop from a burette 0.5 ml of the anticoagulant solution into a series of small bottles. Allow this to dry at room temperature—not in a drying oven or autoclave, since above 80° C. oxalate is changed to carbonate, which has no anticoagulant properties.
- (4) Five milliliters of blood are collected in such a bottle. Before expelling the blood from the syringe, the needle should be removed. Thorough mixing of the oxalate with the blood must be insured by gently inverting the sample a number of times. Blood collected in the above anticoagulant should *not* be used for nitrogen determinations because of the ammonium oxalate present.

71. Volume of Packed Red Cells (Hematocrit)

a. MATERIALS. A Wintrobe hematocrit, pipette and bulb, and a centrifuge are required. The hematocrit (fig. 9) is a glass tube sealed at one end, about 11 cm in length, with an inside diameter of 2.5 mm; on the tube is etched a scale in millimeters and centimeters. The figures at the left of the scale read downward from 0 to 10 cm, and are used for measuring sedimentation rate; those on the right read upward from 0 to 10 cm and are used for measurement of the volume of the packed red cells.

b. PROCEDURE.

- (1) Venous blood is drawn as already described. The blood must be mixed thoroughly by gently inverting the container for 30 seconds or longer; blood is then withdrawn by means of the pipette and bulb; the pipette is passed to the bottom of the hematocrit, and the blood is gradually expelled as the pipette is raised. Care should be taken to leave no bubbles of air in the hematocrit. The tube is filled to the "10" mark.
- (2) The hematocrit may be allowed to stand erect for 1 hour. During or at the end of this time the sedimentation rate of the red corpuscles may be determined; the red cells,



- A. Normal blood.
- B. Simple anemia due to chronic infection.
- C. Chronic posthemorrhagic anemia. The blood plasma is very pale.
- D. Chronic myeloid leukemia. There is a thick layer of white corpuscles and platelets above the red corpuscles.
- E. Pernicious anemia. Note the small amount of packed red corpuscles, the very narrow layer of leukocytes and platelets, and the coloring of the blood plasma due to increased bilirubinemia.
- F. Catarrhal jaundice and slight anemia. In this case the coloring of the blood plasma is due to biliary obstruction rather than to increased destruction.

Figure 9. Hematocrit.

(The appearance of centrifugalized blood in various conditions. (Oxalated venous blood was placed in Wintrobe hematocrit tubes and centrifugalized at 3,000 revolutions per minute for $\frac{1}{2}$ hour.))

100

100

which are heavier than leucocytes and platelets, become separated from them. This step is not essential and may be omitted if the sedimentation rate is not required.

- (3) The hematocrit is centrifugalized until no further packing of the red cells occurs. The time required may be determined by trial with the centrifuge used. The speed of centrifugation must be at least 2,800 rpm. Complete packing occurs at 3,000 revolutions per minute in 30 minutes (speed "10" on an International centrifuge, Type SB, head radius 9 cm). The hematocrit can be placed in a 15 ml metal centrifuge cup without support, but the bottom of the cup should be padded with a rubber cushion or small wad of cotton. The centrifuge must be balanced by placing another hematocrit in a 15 ml cup in the corresponding position on the other side of the centrifuge head.
- (4) Since the hematocrit is a tube of uniform inside bore and flat bottom, the readings may be made directly from the scale on its side. If the hematocrit has been filled exactly to the "10" mark, the upper level of packed red cells multiplied by 10 gives the percentage of packed red cells. If the hematocrit was not filled to "10," then the volume of packed red cells, as read, is divided by the reading at the meniscus made by the blood plasma, and then multiplied by 100.

The volume of packed white cells and platelets (the reddish-gray layer above the packed red cells) affords a rough estimate of the quantity of these constituents. They normally form a layer 0.7 to 1.0 mm in thickness in the hematocrit.

c. If the Wintrobe hematocrit is not available, a 15 ml graduated centrifuge tube can be used. Such a tube, however, requires 15 times as much blood as the Wintrobe tube, and the accuracy of calibrations cannot be easily checked. Tubes of the same diameter as that of the Wintrobe tube can be improvised from glass tubing. (See par. 14).

72. Mean Corpuscular Volume, Hemoglobin and Hemoglobin Content

a. COLOR INDEX. The term "color index" means the amount of hemoglobin in the average red-cell of the patient compared with the normal amount. Thus,

$$\text{Color index} = \frac{\text{hemoglobin (percent)}}{\text{red cells (percent)}}.$$

To express the red-cell count as percent, it is necessary to multiply the first two figures of the count by two. For if the red-cell count is 5,000,000, and the hemoglobin 100 percent,

$$\text{Color index} = \frac{100}{50 \times 2} = 1.$$

A normal color index ranges from 0.85 to 1.15.

b. Since it is not possible to fix a figure for hemoglobin or for red-cell count that is truly representative of the average for men and women as well as for children of different ages, it is better to calculate the hemoglobin content of the average red cell in absolute terms. If the volume of the packed red cells is determined at the same time as the red-cell count and hemoglobin, it is possible to calculate also the volume of the hemoglobin and the hemoglobin concentration of the average red cells, as follows:

- (1) *Mean corpuscular volume (M.C.V.)*. This is the volume of the average red cell of a given sample of blood, and is determined by dividing the volume of packed red cells, expressed in milliliters per 1,000 ml of blood, by the red cell count, expressed in millions. For example, if given blood contains 5,100,000 red cells per cu mm and 44.6 ml of packed red cells per 100 ml of blood, then

$$\text{M.C.V.} = \frac{44.6}{5.1} = 8.7 \text{ cubic microns.}$$

- (2) *Mean corpuscular hemoglobin (M.C.H.)*. This is the amount of hemoglobin, by weight, in the average red cell of the sample of blood. It is determined by dividing the hemoglobin of the blood, expressed in grams per 1,000 ml of blood, by the red-cell count, expressed in millions. For example, if the red-cell count is 5,100,000, and the hemoglobin 15.2 gm per 100 ml, then

$$\text{M.C.H.} = \frac{15.2}{5.1} = 2.98 \text{ micromicrograms.}$$

A micromicrogram is a millionth of a millionth of a gram.

- (3) *Mean corpuscular hemoglobin concentration (M.C.C.)*. This is the proportion of hemoglobin contained in the

Table III. Normal Red-Cell Values

Determination	Male		Female	
	Average	Range of normal	Average	Range of normal
Red blood cells (in millions per cu mm) --	5.4	4.6-6.2	4.8	4.2-5.4
Hemoglobin (in grams per 100 ml blood) --	16	14-18	14	12-16
Volume of packed red cells (in ml per 100 blood).	47	40-54	42	37-47
Mean corpuscular volume (in cubic microns).	Average, both sexes, 87; range of normal, 82-92.			
Mean corpuscular hemoglobin (in micro-micrograms).	Average, both sexes, 29; range of normal 27-31.			
Mean corpuscular hemoglobin concentration (in percent).	Average, both sexes, 34; range of normal, 32-36.			

average red cell of the sample of blood. It is determined by dividing the hemoglobin, expressed in grams per 100 ml by the volume of packed red cells, expressed in cubic centimeters per 100 ml, and multiplying the result by 100. For example, if the hemoglobin is 15.2 gm per 100 ml, and the volume of packed red cells 44.6 per 100 ml of blood, then

$$\text{M.C.C.} = \frac{15.2}{44.6} \times 100 = 34 \text{ percent.}$$

c. IMPORTANCE OF ACCURACY. Simple calculation should make it evident that the value of determinations of the size and hemoglobin content of the red cells depends on the accuracy of the red-cell count, hemoglobin and volume of packed red cells, from which they are calculated. An error in red-cell count of 500,000 can obviously give a misleading value for mean corpuscular volume. Few people realize what the range of error of red-cell counts is in the hands of many technicians. Unless the technical work is of high quality it is better not to attempt to calculate the mean volume and the hemoglobin content of the red cells.

d. NORMAL VALUES. The normal values for mean corpuscular volume, hemoglobin, and hemoglobin concentration are given in table III.

73. Icterus Index

a. This measures the intensity of the color of the blood plasma. Following centrifugation of the blood in the hematocrit tube, the color of the plasma is matched against a series of standards containing arbitrarily fixed concentrations of potassium bichromate. The normal is 5.0 to 7.5 units.

b. The measurement of the icterus index in the hematocrit permits this test to be used as a quick index of the presence of abnormal pigmentation of the plasma, without recourse to the chemical laboratory. If the icterus index is elevated, a van den Bergh test should be carried out.

c. Standard tubes can be purchased, but they can also be easily made by taking glass tubing of about the same bore as the hematocrit, cutting it into lengths of about 12 cm, and sealing one end. The dilutions of potassium bichromate are made from a 1:100 solution, as follows:

<i>Dilution</i>	<i>ml of dichromate stock solution 1 to 100</i>	<i>ml of distilled water containing 2 drops of conc. H_2SO_4 to 100 ml</i>	<i>Icterus index</i>
1:100 -----	10.0	0.0	100
1:133 -----	7.5	2.5	75
1:200 -----	5.0	5.0	50
1:400 -----	2.5	7.5	25
1:500 -----	2.0	8.0	20
1:666 -----	1.5	8.5	15
1:1,000 -----	1.0	9.0	10
1:2,000 -----	0.5	9.5	5

Each of the dilutions is placed in a separate tube, the opening of the tube is sealed, and the tube is marked according to its appropriate icteric value.

74. Sedimentation Test

a. PRINCIPLE. If blood to which an anticoagulant has been added is placed in a narrow tube, the red corpuscles settle out of this suspension, leaving clear plasma above them. In the sedimentation test, the distance that the corpuscles have fallen after a given interval of time is measured.

b. METHOD.

- (1) Venous blood is collected as already described and mixed with the standard oxalate anticoagulant. (See par. 70.)
- (2) A hematocrit tube is filled with blood (par. 71) and is set in a vertical position in an appropriate rack.
- (3) The upper level of sedimenting corpuscles is read at frequent intervals or, more simply, a single reading is made at the end of 1 hour.
- (4) The result is recorded as the number of millimeters that the corpuscles have fallen, the period of time being stated.

c. Certain details must be observed in order to insure accurate and consistent results:

- (1) The hematocrit must be kept in an exactly vertical position during the sedimentation of the red cells, for when the instrument stands at an angle of even 3° from the vertical, significant acceleration of sedimentation takes place. A suitable rack must be prepared or obtained. (See fig. 10.)
- (2) The standard quantity of anticoagulant should be used. Higher concentrations of anticoagulant tend to delay sedimentation.
- (3) The collected blood should be used for the determination of sedimentation rate within 4 hours of its time of collection. Further delay may be associated with increased suspension stability of the blood.
- (4) Since the sedimentation rate increases with increasing temperature, the test should be carried out at a temperature not less than 22° or greater than 27° C. Within this range variations resulting from differences in temperature are small. If the blood used has previously been kept in a refrigerator, it should be permitted to attain the above-mentioned temperature before being used.

d. "CORRECTION" OF SEDIMENTATION RATE. The quantity of corpuscles in suspension obviously influences the stability of the suspension. When anemia is present, the sedimentation velocity is greater, and when polycythemia is present it is slower than that in blood containing a normal quantity of corpuscles. The sedimentation rate as above determined may be "corrected" by cen-

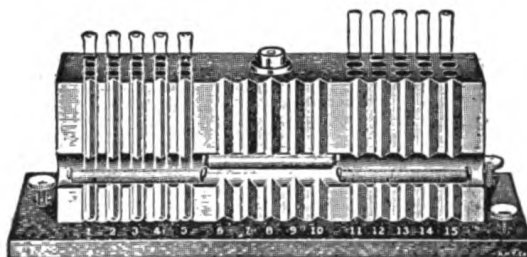


Figure 10. Support for sedimentation (hematocrit) tubes.

A solid block of wood (oak) is provided in front with 15 V-shaped indentations. Each group of 5 indentations is provided with a rubber-covered spring clamp, which holds the tubes in position.

Leveling screws in the base and a spirit level attached to the top of the block make it possible to set the block in an exact horizontal position. Since the V-shaped indentations are cut at a right angle to the base, the tubes set in them are held in vertical position.

The vertical holes drilled in the body of the block provide convenient support for tubes not in use.

trifugalizing the blood in the hematocrit and determining the volume of packed red cells. The "corrected" rate is then found with the aid of a chart. (See fig. 11.)

For correcting the sedimentation rate, find on the chart the horizontal line corresponding to the sedimentation rate for the patient; find also the vertical line corresponding to the volume of packed red cells in the patient's blood. Select the curve lying nearest to the point of junction of the horizontal and the vertical line and follow this to the line corresponding to a volume of packed red cells of 47 ml. The horizontal line at this last point of juncture leads to the corrected sedimentation rate.

e. NORMAL VALUES. Sedimentation velocity is normally greater in women than in men. The chief cause for this is the difference between the sexes in the quantity of red cells. When the sedi-

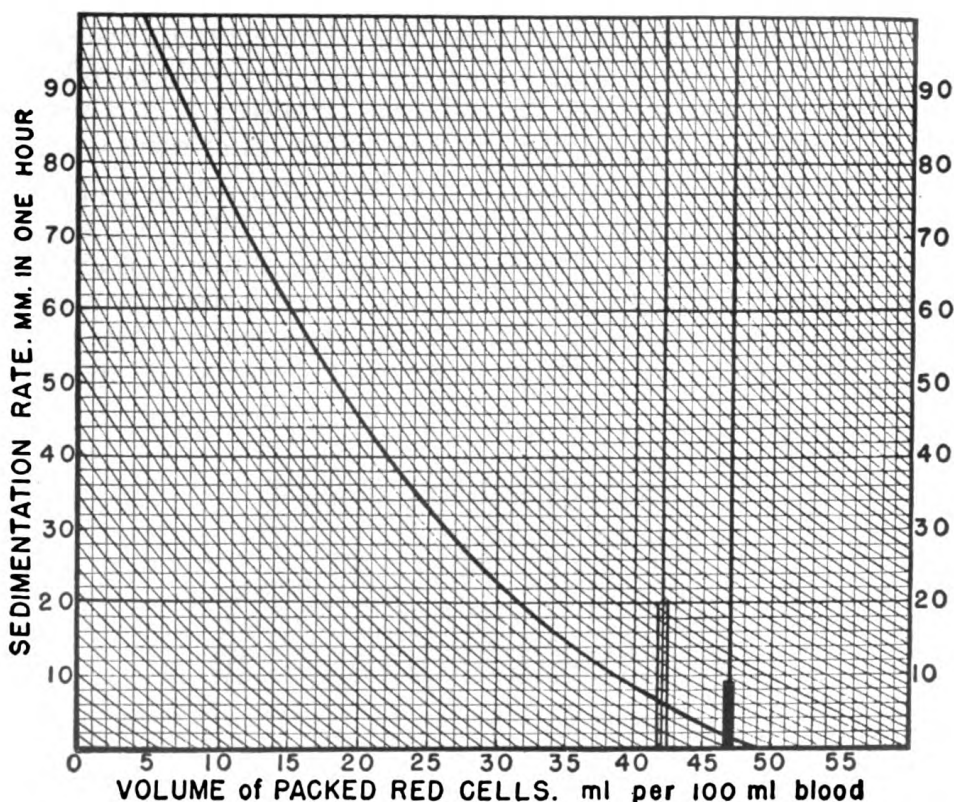


Figure 11. Chart for the correction of sedimentation rate according to volume of packed red cells.

The logarithmic curve on which this chart is based is heavily outlined. The mean normal volume of packed red cells for men (47 ml) and for women (42 ml) are also heavily outlined, and the range of normal sedimentation is represented by solid and open columns for each sex, respectively.

mentation rate is corrected to a volume of packed red cells of 47 ml (the average in men), the sedimentation rates in the two sexes are equal. Normal sedimentation for both sexes may be considered to be from 0 to 6 mm in 1 hour; values of 7 to 10 mm are "border-line." Values between 11 and 15 mm represent slight increases in rate, 16 to 24 mm moderate increases, and 25 mm or greater marked acceleration.

The correction of the sedimentation rate for anemia is only an approximation. Occasionally it may be misleading. Consequently it is necessary to record uncorrected as well as corrected sedimentation rate.

f. ALTERNATIVE METHODS.

- (1) (a) If graduated Wintrobe sedimentation tubes are lacking, ungraduated tubes can be used in a rack arranged for reading the height of the column of red cells against a graduated background. Such a rack and tubes can be improvised in almost any laboratory. (See fig. 12.)
- (b) The graduated surface against which the tubes are held can be prepared most easily by using millimeter graph paper, but if this is not available, plain paper can be ruled by hand with parallel lines at 1-mm intervals, with every tenth line accentuated. Type or draw numerals on the paper in appropriately spaced columns to indicate the millimeters by tens reading downward, but in one column reverse the numerals to read upward. Cement the paper to a glass plate or smooth wooden board, and then cut vertical grooves 1 or 2 mm in width in the paper between the columns of numerals. Place the plate so prepared in a suitable base, with clips to hold the sedimentation tubes against the grooves in the paper. The clips can be made from any light spring metal, either individually or by cutting out a plate (fig. 12), or from wire (paper clips) suitably bent and fixed by screws in the base. The shallow grooves serve to hold the sedimentation tubes perpendicularly; and, in case of the glass plate, permit readings to be made by transillumination if a light is placed behind the plate. The tubes are made from straight plain glass tubing with an inside diameter of 2 to 3 mm and a 1-mm wall, cut in lengths of about 115 mm. Fire-polish one end of each tube and seal the other by just touching it to the margin of a hot

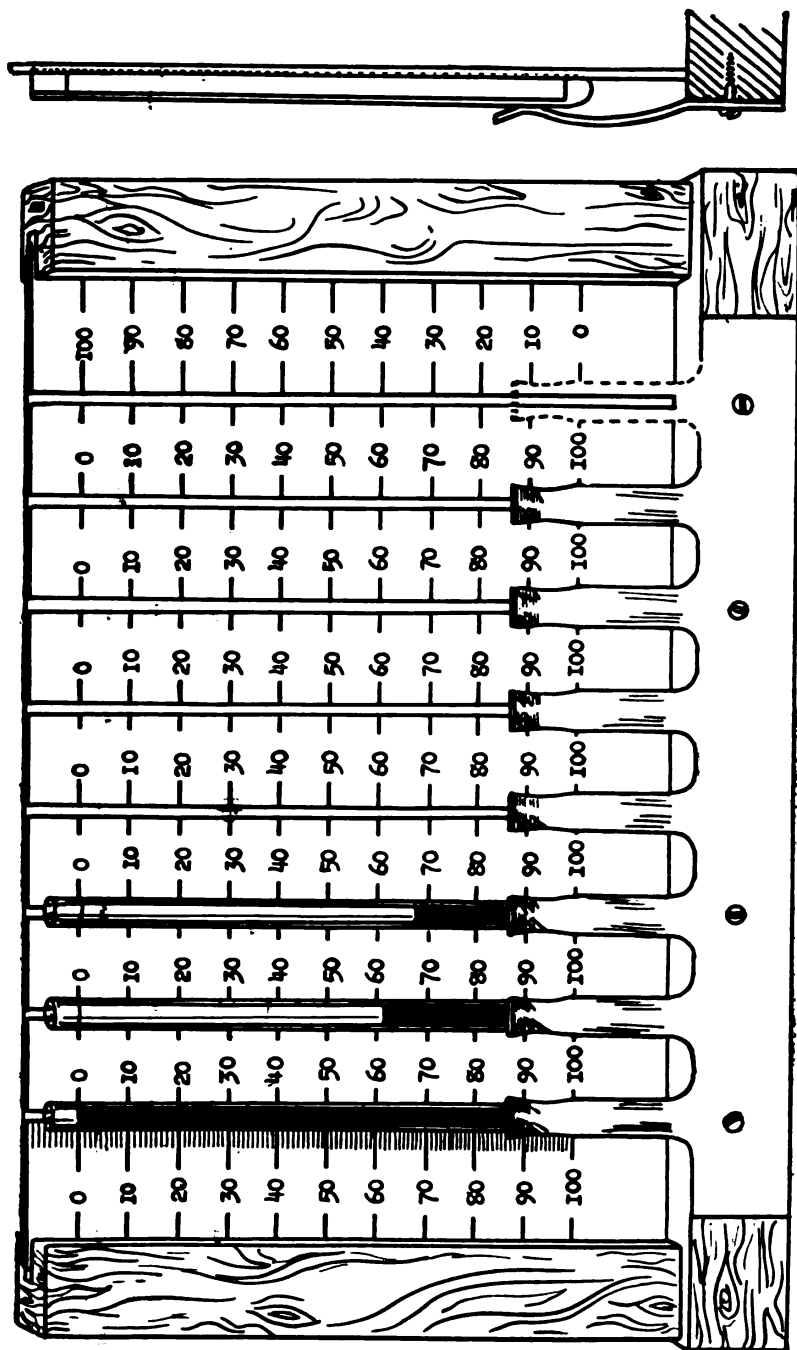


Figure 12. Rack for holding ungraduated sedimentation tubes against a graduated background. The lines for millimeter graduations, shown here in only one column, should be drawn continuously across the background. Frontal view represents three tubes in place; Sagittal section shows a tube held by a spring clip against the graduated background.

flame. By careful rotation of the tube while sealing, the glass can be made to flow to give a flat inner seal. A single graduation mark is scratched or etched on the tube to indicate a column of blood 100 mm in height.

- (c) For the test, fill the tube with blood exactly to the 100-mm mark, using a long-stemmed capillary pipette; then slip the lower end of the tube under the clip, and adjust its height in the vertical groove to place the 100-mm mark on the tube even with the top zero line of the graduated background. Make sure that the rack is so adjusted that the tubes are absolutely perpendicular. Complete the sedimentation readings, as described above, and then centrifuge to pack the cells completely. To read the volume of packed cells, hold the tube against the groove adjacent to the column of numerals reading upward, and read the cell volume directly in percent.
- (2) If proper tubes are lacking, standard laboratory glassware can be used, as follows:
 - (a) Select test tubes from 75- by 10-mm stock, of such caliber that 2 ml of liquid produces a column 55 mm high; etch or scratch the tube at that point.
 - (b) Put exactly 0.2 ml of a 3 percent aqueous solution of sodium citrate in the tube.
 - (c) Fill to the 50-mm mark with blood, and mix by inverting, to avoid air bubbles.
 - (d) Set the tube in the vertical position, and measure the height of the cell column with a millimeter ruler, again recording this height at the end of 1 hour.
 - (e) The normal 1-hour drop is 2 to 8 mm for men and 2 to 10 mm for women.

75. Reticulocyte Counts

a. PRINCIPLE. When red cells in the fresh, undried state are mixed with cresyl blue, a blue reticulogranular network will appear in some of them. Such cells are called reticulocytes. Normal blood contains 0.5 to 1.0 percent reticulocytes, or 5 to 10 per 1,000 red cells.

b. CAPILLARY-TUBE METHOD.

- (1) *Materials.* The following materials are needed.
 - (a) Equipment for finger puncture, or a bottle containing blood.

- (b) Clean glass slides.
- (c) Capillary glass tubes, 2 to 3 mm outside diameter and about 5 cm long.
- (d) Supravital stain, prepared by dissolving 1 gm of brilliant cresyl blue and 5 gm of sodium citrate in 100 ml of distilled water.
- (e) Wright's stain.

(2) *Procedure.*

- (a) Fill a capillary tube half full of the supravital stain.
- (b) Place it in an incubator to dry.
- (c) Fill the tube containing the dried stain half full of blood.
- (d) Tilt the tube back and forth until all the dried stain is dissolved by the blood.
- (e) Allow the tube to stand for 15 to 30 minutes.
- (f) Place the contents of the tube on a clean glass slide and make a thin smear.
- (g) Counterstain with Wright's stain, and examine under the oil-immersion lens.
- (h) Count 1,000 red cells, noting the number of reticulo cytes.

c. WET-FILM METHOD.

(1) *Materials.* The following materials are required:

- (a) Equipment for finger puncture, or a bottle containing blood.
- (b) Clean cover glasses and slides.
- (c) Stain, prepared by dissolving 1.0 gm of brilliant cresyl blue and 0.4 gm sodium citrate in 100 ml physiologic saline solution.

(2) *Procedure.*

- (a) Place a small drop of blood in the center of a cover glass, and to this add about twice as much stain.
- (b) Invert this on a slide, drawing up the excess of stain and blood at the edges of the cover glass by capillary attraction with a piece of gauze. (The thinner the preparation, the easier the count.)
- (c) Ring the cover glass preparation with vaseline, and let it stand for 5 to 10 minutes.
- (d) Examine under oil immersion lens.
- (e) Count, 1,000 R.B.C., 200 in the center and 200 in each of the four corners of the cover glass preparation.
- (f) Note the number of red cells that have purplish granules or strands of reticulum.

- (g) The number of cells with granules or reticulum divided by 10 is the percent of reticulocytes.
- (h) Normal counts: 2 to 20 reticulated R.B.C. per 1,000 or 0.2 to 2.0 percent.

d. ALTERNATIVE METHODS.

- (1) A drop of blood on a clean cover glass is touched with cresyl blue (1 percent solution in physiologic saline solution) on the end of a toothpick so that the blood becomes blue. A blood smear is then made in the usual manner, stained with Wright's stain, and mounted on a slide.
- (2) Cover glasses may be prepared by pulling smears of a 0.3 percent *alcoholic* solution of cresyl blue on scrupulously clean cover glasses. The excess dye is drained off by allowing the cover slip to stand tilted on one edge, and the preparation is allowed to dry in this position. The stained cover glasses are then gently polished with clean linen to remove the excess of stain. On these prepared cover glasses, blood smears are made in the usual manner from the finger-prick blood of the subject, and counterstained with Wright's stain after drying.
- (3) Cover slips prepared as described above may be used in making fresh blood preparations.

e. COMMENTS.

- (1) Counting is greatly facilitated by putting into the ocular of the microscope a screen of stiff paper or cardboard in the center of which a small rectangular hole has been cut. In this way the size of the field is reduced.
- (2) Red cells covered with precipitated stain should not be confused with reticulocytes.

76. Platelet Counts

No method is completely satisfactory because platelets tend to agglutinate and to disintegrate readily. When blood is obtained from the finger it is important that the finger be perfectly clean. If the skin is rough, a thin coat of vaseline may be applied and the puncture made through this. If venous blood is employed, the needle should be extremely sharp, and the syringe scrupulously clean.

a. DIRECT METHOD.

- (1) *Materials.* The following materials are needed:
 - (a) Platelet solution which is prepared as follows:

Sodium citrate	3.8 gm
Formaldehyde (neutral 40 percent solution) -	0.2 ml

Brilliant cresyl blue -----	0.1 gm
Distilled water -----	100 ml

This should be kept in a glass-stoppered bottle in an ice box when not in use. It must be filtered each time before use, and blank counts on the solution should be made at intervals, to discover the presence of bacteria, molds, or plateletlike bodies. Sometimes it will be found that solutions which have stood for a time cause hemolysis of red cells. Such solutions should be discarded. The hemolysis is usually produced by formic acid, formed by the oxidation of formaldehyde.

(b) Red-cell pipette, and counting chamber.

(2) *Procedure.*

(a) A red-cell pipette is filled with the platelet solution, which is immediately expelled. A film of fluid, which tends to prevent the platelets from sticking to the glass, is thus left inside the pipette.

(b) Blood is drawn to the 0.5 mark, and platelet solution is then drawn to the 101 mark.

(c) The pipette is mixed as for a red-cell count, and the counting chamber is filled in the usual manner. If not examined immediately, the counting chamber is placed in a petri dish or similar container in which has been placed a small, moistened piece of filter paper, to prevent evaporation.

(d) Platelets appear as small, round or oval, refractile, slightly bluish bodies, lying singly or in clumps. Usually they are one-eighth to one-fourth the size of red cells.

(e) The count is made under the high-dry lens in the central, finely ruled portion of the counting chamber. All platelets seen in the 25 groups of 16 small squares (new type chamber) are counted, and this value is multiplied by 2000 to give the number per cubic millimeter.

(f) The pipette and counting chamber must be scrupulously clean and particles of dust or precipitated stain should not be mistaken for platelets.

(3) The normal platelet count with this method is 200,000 to 300,000 per cubic millimeter.

b. INDIRECT METHOD (FONIO'S).

(1) *Materials.*

The following materials are required:

(a) Equipment for finger puncture.

(b) Equipment for red-cell count.

- (c) Clean slides.
- (d) A 14 percent aqueous solution of magnesium sulfate.
- (e) Wright's stain.
- (2) *Procedure.*
 - (a) Puncture the finger, and place a drop of the magnesium sulfate solution over the puncture before the blood begins to flow.
 - (b) Allow the blood to flow into the drop until the proportion is about 1 part of blood to 4 parts of solution.
 - (c) Place this drop on a clean slide and make a thin smear.
 - (d) Stain the smear with Wright's stain, and examine under the oil-immersion lens.
 - (e) Count 1,000 red cells, noting the number of platelets.
 - (f) Calculate the number of platelets per cubic millimeter by multiplying the number seen in counting 1,000 red cells by the red-cell count divided by 1,000.
- (3) The normal platelet count by this method is 200,000 to 500,000 per cubic millimeter.

77. Bleeding Time

The bleeding time is the time that it takes the blood to stop flowing from a measured cut in the finger or ear.

a. MATERIALS. Finger-puncture equipment, filter paper, and a watch are needed.

b. PROCEDURE.

- (1) Make a finger-puncture or, more conveniently, an ear-lobe incision sufficiently deep so that a drop of blood 2 mm in diameter appears without any squeezing; note the time of incision.
- (2) Take a piece of smooth filter paper and blot off the drop every 30 seconds (no manipulation of the part is permitted). Just the tip of the drop should be touched with the filter paper.
- (3) The interval between the time of incision and that of the last drop is considered the bleeding time.
- (4) The normal bleeding time is 1 to 3 minutes. When bleeding continues longer than 10 minutes, the bleeding time is seriously prolonged.

78. Coagulation Time (Howell Method, Modified by Lee and White)

a. PRINCIPLE. Venous blood is placed into tubes of standard diameter and the time at which the tubes can be inverted without

spilling the blood is noted. Errors in technic tend to hasten coagulation.

b. **MATERIALS.** Venipuncture equipment, three Wasserman tubes, and a watch are required.

c. **PROCEDURE.**

- (1) The sterile syringe, with needle attached, is rinsed out with sterile physiologic salt solution, and all the air expelled, so that the dead space in the needle and tip of the syringe is filled with salt solution and not with air, since the latter tends to hasten coagulation.
- (2) The needle must enter the vein cleanly at the first attempt, since an admixture of tissue juice hastens coagulation.
- (3) The needle must fit tightly, and care should be exercised in filling the syringe not to use too vigorous suction, so that no air bubbles pass through the blood.
- (4) Use test tubes of about 8 mm diameter, and place about 1 ml of blood in each.
- (5) Three tubes should be set up; one is tilted slightly at 30-second intervals to test for coagulation, the others being allowed to stand undisturbed until coagulation has occurred in the first tube. Then the second tube, and finally the third tube is tilted until coagulation has occurred.
- (6) The time is counted from the time the blood enters the syringe until the blood in the last tube has coagulated.
- (7) The normal coagulation time is 6 to 12 minutes. If the coagulation time seems to be prolonged, it is best to carry out a control test with blood from a person known to be normal.

79. Clot-retraction Time

a. A tube in which the coagulation time was determined should be set aside to observe the length of time required for the clot to retract, the clot being gently loosened from the sides of the tube.

b. Observe at the end of each hour for 6 hours, and at intervals of 6 to 12 hours thereafter.

c. Record the elapsed time until the clot has retracted and a clear area of serum has become visible.

d. Under these conditions the clot normally retracts completely within 18 to 24 hours after it is formed. Delay in retraction, or failure of the clot to retract, is usually associated with a decrease in platelets.

80. Prothrombin Time

a. QUICK'S METHOD.

(1) *Principle.* The method is essentially an improvement on Howell's prothrombin test; tissue extract (brain emulsion) is added to the plasma to eliminate differences in clotting time as the result of variations in the thromboplastin content. The test is carried out at 37° C. and a fixed amount of calcium is added.

(2) *Materials.* The following materials are needed:

- (a) *M/10 sodium oxalate solution.* This is made by dissolving 1.34 gm of anhydrous, pure sodium oxalate in 100 ml of distilled water.
- (b) *M/40 calcium chloride solution.* This is made by dissolving 0.28 gm of anhydrous, chemically pure calcium chloride in 100 ml of distilled water.
- (c) *Thromboplastin.* This is made from rabbit brain and can be purchased commercially. To prepare thromboplastin locally, the brain of a freshly killed rabbit is freed of the larger superficial blood vessels and macerated under acetone. The acetone is poured off and the process twice repeated with fresh acetone. The granular residue is then spread in a thin layer on a piece of plate glass and thoroughly dried at 37° C. It should be evaluated for thromboplastic activity before storage by the method employed in the routine test. The dried thromboplastin may be preserved in lots of 0.3 gm in small glass vials that are evacuated and sealed. An alternative method of preservation which insures stability of the thromboplastin for 6 to 9 months, is to mix 0.3 gm of the dried thromboplastin with 4.9 ml of 0.85 percent sodium chloride solution and 0.1 ml of 1.34 percent sodium oxalate solution. The mixture is added to a suitably stoppered test tube or vial and placed in the freezing compartment of the refrigerator. It is left in the frozen state until needed.

To prepare the thromboplastin solution for use:

1. Frozen extract is placed in an incubator or water bath at 37° C. for 10 minutes and centrifuged slowly to throw down the coarse particles.
2. 0.3 gm of the dried powder extract is mixed with 5.0 ml of 0.85 percent sodium chloride solution containing 0.1 ml of 1.34 percent, sodium oxalate solution, and incubated for 15 minutes at 50° C. The mixture is centrifuged as above.

The milky supernatant fluid is used for the test in each instance.

- (d) Venipuncture equipment.
- (e) Chemical pipettes, 1 ml size, measuring 0.1 ml quantities.
- (f) Dry, clean test tube (13 by 100 mm).
- (g) Water bath at 37°.
- (h) Stop watch or clock.
- (i) Centrifuge.

(3) *Procedure.*

- (a) Four and one-half milliliters of blood, withdrawn rapidly and with special precaution to avoid trauma, is promptly and thoroughly mixed with 0.5 ml of M/10 sodium oxalate and centrifuged at low speed for 5 minutes.
- (b) Of this plasma, 0.1 ml is transferred to a dry, clean test tube and mixed gently with 0.1 ml of thromboplastin solution; the mixture is warmed in the water bath.
- (c) One-fourth of a milliliter of M/40 calcium chloride is added, and the tube quickly shaken.
- (d) A stop watch or clock is started at the instant the calcium chloride is added, and the exact time required for the formation of a firm, semisolid clot is recorded.
- (e) In order to determine the second at which the clot is developed, the tube should be tipped every few seconds.
- (f) The clotting of normal plasma varies, according to the activity of the thromboplastin used, between 10 and 25 seconds, and in reporting the result, the clotting time of normal plasma should always be given. Spontaneous bleeding ordinarily does not occur until the value becomes as high as 40 or more seconds.

b. BEDSIDE METHOD (ZIFFREN ET AL.).

(1) *Materials.* The following materials are required:

- (a) Thromboplastin solution, as prepared above.
- (b) Serologic test tubes (10 by 75 mm), marked on the sides to indicate 1 ml.
- (c) Venipuncture equipment.
- (d) Stop watch.

(2) *Procedure.*

- (a) Place 0.1 ml of thromboplastin into a test tube.
- (b) Draw blood into a clean, dry syringe, and place enough blood into the test tube to reach the 1.0 ml mark.
- (c) Invert the tube over the finger once or twice, then tilt

gently every second or two, noting the time when clotting takes place.

(d) Comparison should be made with the time required for the clotting of normal blood treated in the same manner.

(e) The results are expressed as percentages of normal, the clotting time of normal blood being divided by that of the patient's blood and the quotient being multiplied by 100.

(3) *Precautions.*

(a) It is best to use a grade of thromboplastin that causes clotting in 25 to 50 seconds. If clotting is more rapid than this, accurate measurement of time is difficult.

(b) The vein should be punctured quickly, and the blood should be transferred promptly from the syringe to the test tube.

81. Fragility Test

a. PRINCIPLE. Venous blood is mixed with various strengths of hypotonic sodium chloride solution, and the strengths of the solutions in which hemolysis begins and is complete are noted.

b. MATERIALS. The following materials are needed:

(1) Venous blood, collected with anticoagulant as in par. 70. p. 59.

(2) A 0.75 percent aqueous solution of sodium chloride made from salt that has been weighed *after* it has been dried.

(3) Test tube rack, eighteen 10- by 100-mm test tubes, two 5-ml pipettes calibrated to 0.1 ml and 1-ml pipette calibrated to 0.1 ml.

c. PROCEDURE.

(1) Place test tubes in rack and label with even numbers, from left to right, starting with "48," and ending with "14."

(2) Into the first tube, measure 4.8 ml of sodium chloride solution, into the second 4.6 ml and so on in amounts corresponding to the number of the tube.

(3) To each tube add the amount of *distilled water* required to bring the contents of each test tube to 5 ml, that is, 0.2 ml into the first tube, 0.4 ml into the second, etc.

(4) Mix the contents of each tube thoroughly.

(5) Exactly 0.2 ml of well-mixed blood is measured into each tube, and the tubes are gently shaken to insure perfect mixing.

- (6) The tubes are set aside for at least 2 hours, preferably overnight, in a refrigerator, and then the point of beginning hemolysis and the point of complete hemolysis are read.
- (7) The first tube showing a tinge of red in the supernatant fluid indicates the point of beginning hemolysis. The first point at which no corpuscular residue is seen at the bottom of the tube, or no clouding occurs on shaking the tube, is the point of complete hemolysis.
- (8) The strength of salt in each tube is determined by multiplying the number of the tube by .015.
- (9) If jaundice is present, a control tube is prepared in which 0.2 ml of blood is placed in 5 ml of physiologic saline solution.
- (10) If larger numbers of tests must be performed, time can be saved by preparing stock solutions containing the proper strengths of sodium chloride. If these are used, 5.0 ml of each solution is pipetted into the desired tube of the series.

d. INTERPRETATION.

- (1) Hemolysis in normal blood begins at 0.45 to 0.39 percent, and is complete at 0.36 to 0.30 percent.
- (2) Unless hemolytic jaundice is suspected, it is not necessary to prepare all 18 tubes, since the strengths of saline in the first few tubes are high. Usually the first 4 tubes may be omitted, for even then the first tube ("40") contains 0.60 percent sodium chloride. Similarly the last 2 tubes ("16" and "14") may usually be omitted.

Section II. HUMAN BLOOD GROUPS

82. Isoagglutinogens and Isoagglutinins

Two isoagglutinogens in human red blood cells and two corresponding isoagglutinins in human plasma determine four blood groups. The agglutinogens are designated A and B, and the blood groups are named according to the presence or absence of these agglutinogens in the red cells. Group O, pronounced as the letter "O," indicates the absence of both agglutinogens, group A the presence of agglutinogen A, group B the presence of agglutinogen B, and group AB the presence of both agglutinogens. When an agglutinogen is present in the blood cells the corresponding agglutinin must be absent from the serum, whereas if an agglutinogen is absent from the cells the corresponding agglutinin is always

present in the serum. The agglutinins are designated: anti-A or alpha (α) and anti-B or beta (β). The following table shows the relation between agglutinogens and agglutinins:

Table IV. Relation between Agglutinogens and Agglutinins

Group	Agglutinogens in cells	Agglutinins in serum or plasma
O-----	None-----	Anti-A and anti-B
A-----	A-----	Anti-B
B-----	B-----	Anti-A
AB-----	A and B-----	None

83. Nomenclatures

The use of different systems for designating the blood groups, as successively recommended by Landsteiner, Jansky, and Moss, formerly led to much confusion. That confusion is now eliminated by the universal adoption of the system called the International Nomenclature. The following table shows the relation between the various nomenclatures, but the use of numbers for designating the blood groups is to be discouraged.

Table V. Blood Group Designations

Nomenclature			Approximate distribution among white individuals in U. S. A. (Percent)
International	Jansky	Moss	
O-----	I-----	IV-----	44
A-----	II-----	II-----	38
B-----	III-----	III-----	14
AB-----	IV-----	I-----	4

84. Application

a. The major practical application of blood grouping is in its relation to blood transfusion. Recipient and donor should belong to the same blood group. (See par. 92.) Transfusions are given to replace lost blood and to combat shock, and in the treatment of certain blood diseases, anemias, and infections.

b. Blood grouping is an essential aid to the selection of donors for transfusion. However, it should not be relied on solely except when the emergency is so great that cross-matching cannot also be carried out. It must always be remembered that even if the donor and recipient belong to the same group, intragroup incom-

patibility may exist, and this cannot be detected by simple blood grouping. Errors in grouping will occur if weak or deteriorated grouping serums are used. Furthermore, the blood group designated on identification tags should be accepted only as a preliminary grouping to simplify selection of donors for regrouping and cross-matching. Only in extreme emergency should blood grouping alone be considered sufficient for selection of donors for transfusion. (See pars. 91 and 92.)

c. Transfusions of incompatible blood may result in dangerous or even fatal reactions. This is due to the interaction between substances in the plasma (issoagglutinogens or isohemolysins and corresponding factors in the red cells (isoagglutinogens). This causes the cells to clump (agglutinate) or dissolve (hemolyze), or both. This danger is greatest if the red cells of the donor are agglutinated (or hemolyzed) by the plasma of the recipient. When this set of conditions is found to exist, the donor must never be used. If, however, the test shows only that the cells of the recipient are agglutinated by the plasma of the donor, there is far less danger. Under such conditions thousands of transfusions have been carried out successfully because the donor's plasma is generally diluted in the recipient's circulation beyond its agglutinating strength. Also, the donor's blood is usually transfused so slowly that there is time for its agglutinins to be rendered harmless. As a matter of fact, the transfusion of such bloods is dangerous only when the titer of incompatible isoagglutinins in the donor's blood is exceptionally high, and especially so if the patient to be transfused is very anemic. Dangerously high-titered isoagglutinins can be detected by a simple procedure outlined in paragraph 92b.

85. Universal Donors and Recipients

a. The donor should belong to the same group as the recipient except that, as previously noted, at times proved group O persons may act as universal donors and patients of group AB as universal recipients. (See par. 92.)

b. As indicated in table IV, the plasma of AB bloods does not contain either anti-A or anti-B agglutinins. Therefore, the red cells of any donor, regardless of their agglutino-gen content, are compatible for recipients belonging to this group. It is for this reason that group AB patients are designated universal recipients; such recipients can, as a rule, safely receive blood from donors belonging to other groups.

c. It will also be seen from the table that the red cells in group O blood do not contain either agglutino-gen A or B. Therefore, the

red cells in these bloods are not acted upon by incompatible iso-hemagglutinin in the plasma of recipients belonging to any of the three other groups. It is on this basis that persons of group O are designated universal donors; they can serve as donors to patients of any of the other three groups' and, of course, also to patients of the same group.

86. Determination of Blood Groups

a. REAGENTS AND EQUIPMENT.

- (1) The only reagents required for blood grouping are specific and potent agglutinating serums. (See par. 95.) With satisfactory serums fairly complete agglutination should be visible to the naked eye within 15 to 20 seconds. The activity of grouping serums should be checked at weekly intervals against known A and B cells to avoid the use of deteriorated serums that may become too weak to group all bloods properly.
- (2) The equipment required is as follows: glass slides; wooden applicators or toothpicks; wax pencils; capillary pipettes fitted with rubber bulbs or hypodermic syringes fitted with 24-gauge needles; small test tubes (8 x 75 mm); 4 percent sodium citrate; physiologic saline solution (0.85 percent sodium chloride); microscope and centrifuge.
- (3) If a microscope or centrifuge is not available, grouping and cross-matching can be carried out by the macroscopic slide technic outlined in paragraph 87.

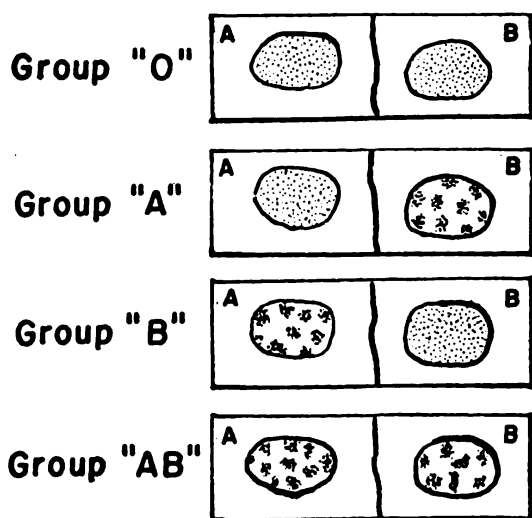
b. CHOICE OF METHODS. Blood grouping can be performed by the slide method or by the centrifuge test tube method. Grouping of large numbers of individuals under military conditions is best done by the slide method but the test tube centrifuge method is recommended whenever practical, since it is considered somewhat more sensitive and rouleaux formation is avoided. For mass grouping technic, see paragraph 90.

c. TERMINOLOGY. The terminology of grouping serums may confuse the beginner. Serums which agglutinate A cells are designated anti-A and serums which agglutinate B cells are designated anti-B. Anti-A serums are obtained from group B individuals and may be designated as B serums; anti-B serums are obtained from group A individuals and may be designated as A serums. Thus, grouping serums may be designated as B or anti-A and as A or anti-B. The terms anti-A and anti-B are considered preferable and less confusing.

87. Slide Method

The test is made as follows:

- a. Divide a slide equally with a wax pencil.
- b. Place the subject's initials or number in the lower right-hand corner of the slide, the letter "A" in the upper left-hand corner, and the letter "B" in the upper right-hand corner, as indicated in figure 13.
- c. The blood should be collected preferably by venipuncture (5 ml) ; if this is not feasible, it may be obtained by deep puncture of a finger or earlobe (0.5 to 1.0 ml). Cleanse the site from which the blood is to be obtained with alcohol, and allow to dry. The blood is placed in tubes containing citrate solution in an amount approximately equal from 1/5 to 1/10 of the volume of the blood drawn.
- d. A red-cell suspension is prepared by mixing 1 drop of the citrated blood with about 1 ml of saline solution. With normal blood this gives a cell suspension of approximately 2 percent. With anemic patients, more blood should be added, to make a suspension matching in color to that of the donor's. Mark the tube containing the recipient's blood "RC" (recipient's cells), and that containing the donor's blood "DC." Save the remainder of the blood collected for cross-matching.
- e. Place 1 drop of cell suspension on each half of the marked glass slide.
- f. Place 1 drop of A (anti-B) serum on the left side of the slide and 1 drop of B (anti-A) serum on the right side of the slide.
- g. Mix well with a wooden applicator or toothpick (separate end for each side), rock the slide manually 5 to 10 seconds to in-



(Fine dots represent no clumping (negative reaction). Massed dots represent agglutination (positive reaction).)

Figure 13. Blood groups.

sure thorough mixing, then allow to stand for 5 to 10 minutes, tilting a few times about once every minute.

h. In warm climates where the slide preparation is apt to dry up, it should be kept in a moist chamber during the period of observation. (A moist chamber can be made by placing pledgets of wet cotton under petri dishes or glass trays. If the use of a moist chamber is impracticable, the addition of 1 drop of saline solution to each side of the slide after about 5 to 10 minutes' standing usually prevents drying).

i. The reactions are read with the naked eye, and negative reactions under the low power of the microscope, if one is available. In a positive reaction the cells are stuck together in clumps usually visible to the naked eye. The interpretation of the grouping tests is shown in figure 13 and in the left-hand half of the list of reactions.

Table VI. Blood Group Reactions

Clumping of unknown cells with known serums		Agglutinogens in cells	Clumping of known cells with unknown plasma		Agglutinins in plasma	Group
Anti-B A	Anti-A B		A	B		
—	—	None	+	+	Anti-A and anti-B	O
—	+	A	—	+	Anti-B -----	A
+	—	B	+	—	Anti-A -----	B
+	+	A and B	—	—	None -----	AB

+ Clumping. — No reaction.

88. Centrifuge Test Tube Method

a. Label one small test tube (Kahn tube) "anti-A" and another "anti-B," and to the respective tubes add one drop of anti-A serum and one drop of anti-B serum.

b. Place one drop of the 2 percent suspension of cells in each of the two small tubes and mix.

c. Place one drop of salt solution in each tube. (The addition of salt solution does not decrease the sensitivity but does decrease chance for rouleaux formation.)

d. Centrifuge both tubes at 1,000 rpm for 2 minutes.

e. Agitate the tubes gently in a good light and observe for clumping of the red cells. Check all negative readings with the microscope.

f. Agglutination with the anti-A serum indicates group A; agglutination with anti-B serum indicates group B; agglutination in neither tube indicates group O; and in both tubes, Group AB.

89. Confirmation of Grouping by Testing of Plasma

When time permits, and preferably as a routine, the following confirmatory test on the plasma of the individual being grouped should be carried out. This is essential for the certification of universal donors. (See par. 92.)

a. Divide a slide in halves with a wax pencil and mark the left side "AC" (group A cells) and the right side "BC" (group B cells).

b. On the left half of the slide place a drop of fresh known group A cell suspension; on the right half place a drop of known group B cell suspension.

c. Add 2 drops of plasma to each side of the slide, and mix each by stirring with a separate applicator or toothpick.

d. Observe the slide for at least 20 minutes, tilting it back and forth at 2- or 3-minute intervals, and then examine for agglutination.

- (1) If it is difficult to distinguish between true agglutination and rouleaux-formation (par. 93), stir again with an applicator. This will usually break up rouleaux into a uniform suspension.
- (2) If hemolysis occurs this is due to complement present in the fresh plasma. The complement in the plasma must be inactivated by heating at 56° C. for 20 to 30 minutes and the test repeated.
- (3) The scheme of identification of blood groups from the reaction of unknown plasma and known cells is given in the right-hand half of table VI.

90. Mass Groupings

a. When large numbers of persons are to be grouped in a relatively short space of time (500 or more per day), certain modifications of the technic just described are necessary, which are as follows:

- (1) For the sake of expediency the test should be done on glass slides.
- (2) The slides should have the left and right halves marked "A" and "B" in advance, as indicated in paragraph 87b.
- (3) A team of three persons should work simultaneously at a table. The personnel to be grouped file past one by one. Accurate blood grouping can be done at the rate of about 60 to 90 per hour. The three members of the grouping team may be designated as X, Y, and Z.
- (4) X cleanses and punctures the finger as described above.

(See pars. 60 and 87c). He places a small drop of whole blood, the size of a pinhead, on each side of one of the slides by touching the drop to the slide as it forms (the use of too large a drop may obscure and delay the agglutination reaction.) He numbers the slides serially with a wax pencil. To the left-hand slide he adds 1 drop of A (anti-B) serum, and to the right-hand half 1 drop of B (anti-A) serum. He mixes each with a toothpick or applicator, rocks the slide for 10 to 20 seconds, and makes a preliminary reading, which is recorded by Z.

- (5) The individual being grouped has, in the meantime, passed to member Y, who independently repeats the test, but uses grouping sera from different bottles. He also makes a preliminary reading, which is likewise recorded by Z.
- (6) Both X and Y pass their slides to Z, who rocks them a few times—about once every 5 minutes—and retains each slide until 30 minutes have elapsed. This is important to insure that weak subgroups of A, particularly of AB, do not escape detection.
- (7) In case of discrepancy between the results of the tests carried out by X and Y, or between the readings and the group given on the person's identification tag, if any, he should be recalled for regrouping later, when enough blood is taken for plasma, as described in paragraphs 50, 51, 87c. Then both cells and plasma should be tested. (See pars. 87 and 88.)
- (8) Member Z discards the old slides for washing after the lapse of 30 minutes, at the same rate that new ones accumulate. He keeps records in a book ruled with seven columns; individual's name and number, group from identification tag (if any), preliminary reading by X, final reading of X's slide by Z, preliminary reading by Y, final reading of Y's slide by Z, and final grouping.

b. The method described above is reliable only if the serums are of high potency, the size of the drop of blood is small (about the size of a pinhead), and the grouping serums are added before the blood has a chance to dry.

91. Cross-Matching

a. GENERAL. After a donor belonging to the same blood group as the patient has been selected, the cross-matching test must be performed before the transfusion is given. This test will reveal any mistakes in blood grouping and will also usually indicate the

presence of unusual agglutinins, such as Rh agglutinins which may render the donor's blood incompatible even though of the same blood group. Some Rh agglutinins are not active in saline and for this reason cross-matching should be done using cell suspensions in serum, plasma or bovine albumin rather than in saline. Rh agglutinins act best at 37° C. and the tests should be performed at this temperature. Either the tube test or the slide test may be used but most workers feel that the tube test is preferable.

b. TUBE TEST.

- (1) Draw 3-5 ml of blood from both donor and recipient as for a Kahn test and separate the serum from the clot. Inactivate the serum at 56° C. for 30 minutes when time permits.
- (2) Place 1 ml of recipient's serum in a small test tube (Kahn tube) and add sufficient cells from the recipient's clot to give a 2 percent suspension. Cells may be loosened from the clot by shaking. One small drop of the resulting cell suspension in 1 ml of serum will give approximately a 2 percent suspension. Technicians must familiarize themselves with the appearance of 2 percent suspensions by preparing known 2 percent suspensions for comparison. One drop of normal whole blood in 1 ml of fluid is approximately a 2 percent suspension.
- (3) Prepare a similar 2 percent suspension of donor's cells in donor's serum.
- (4) Place 1 drop of recipient's serum in a tube labeled RS/DC and 1 drop of donor's serum in a tube labeled DS/RC. Add 1 drop of donor's cell suspension to the first tube and 1 drop of recipient's cell suspension to the other tube and proceed as in (6) below.
- (5) When the cross-match is performed with donor's blood from a blood bank and only citrated blood is available, a 2 percent suspension of cells may be prepared by adding 1 drop of whole blood to 1 ml of 20 percent bovine albumin. (Add 10 ml of sterile 0.85 percent saline solution to the 20 ml of 30 percent bovine albumin in the vial.) Washed cells are preferable when the blood is over five days old. Add several drops of citrated whole blood to 1 or 2 ml of 0.85 percent saline solution, centrifuge and decant the saline. Add 1 drop of these washed, packed cells to 1 or 2 ml of 20 percent bovine albumin. If bovine albumin is not available, AB serum may be used. Place 1 drop of recipient's plasma in a small test tube labelled RP/DC and 1 drop of donor's plasma in a

small test tube labelled DP/RC. Add 1 drop of donor's cell suspension to the first tube and 1 drop of recipient's cell suspension to the second tube.

- (6) Centrifuge for 1 to 2 minutes at 500 to 800 rpm and if no agglutination is evident, place in a water bath at 37° C. for 30 minutes and centrifuge again at 500 to 800 rpm for 1 to 2 minutes. In emergency, the period of incubation may be shortened to 5 or 10 minutes but it is safer to use the longer period.
- (7) Hemolysis is indicated by reddish discoloration of the supernatant serum. This will not occur if the serum has been inactivated and rarely occurs when plasma is used. Examine over a concave mirror for agglutination by shaking the tube gently and observe for coarse clumps. Check by pouring the contents on a slide and examine with a microscope. Rouleaux formation may be broken up by adding 0.85 percent sodium chloride. Agglutination or hemolysis of donor's cells by recipient's serum or plasma indicates that the donor is not suitable. Agglutination or hemolysis of the recipient's cells by the donor's serum or plasma should not occur if the recipient and donor are of the same blood group. If it does occur, an error in grouping is likely. Agglutination will invariably occur if group O blood is used for group A, B, or AB recipients. Under these circumstances, the donor may be used if the rate of transfusion is slow enough to allow rapid dilution of the donor's blood and providing the titer of incompatible agglutinins in the donor's blood is not excessively high. It is always preferable that the donor be of the same blood group as the recipient. (Par. 84c).

c. SLIDE TEST.

- (1) In order to demonstrate Rh antibodies as well as anti-A and anti-B agglutinins, by the slide method, it is necessary to use a 40 to 50 percent suspension of cells in serum, plasma or in 20 percent bovine albumin rather than the usual 2 percent suspension of cells in saline. Whole citrated blood may be used or a 40 to 50 percent suspension of cells may be made in their own serum by adding with a pipette 2 drops of blood from the clot to 2 drops of serum. An approximate 40 percent suspension may also be made by pouring off most of the serum from the clotted, centrifuged blood and shaking the remaining clot and serum. If the donor's blood is obtained from a blood bank and is mixed with a preservative, centrifuge

a sample and add 2 drops of packed cells to 2 drops of 20 percent bovine albumin or to 2 drops of serum from AB blood.

- (2) Divide a clean slide as for the grouping test and mark the left side "DS/RC" and the right side "RS/DC."
- (3) Place 1 drop of the donor's serum (DS) or plasma on the left side and 1 drop of the recipient's serum (RS) or plasma on the right, using different capillary pipettes (or syringes and needles) for each transfer.
- (4) Add 2 drops of the recipient's cell suspension or whole citrated blood (RC) to the drop of donor's serum or plasma and 2 drops of the donor's cell suspension or whole citrated blood (DC) to the drop of the recipient's serum or plasma.
- (5) Mix well with a wooden applicator or toothpick (separate end for each side), rock the slide to insure even mixing and observe over a period of 5 minutes with occasional rocking. Since Rh agglutinins act best at 37° C., the slide should be placed over an illuminated viewing box during the period of observation. A flat bottle filled with water and kept in a 37° C. incubator improvises a useful warm slide.
- (6) Rouleaux formation may confuse the inexperienced technician. Cells are arranged in rows resembling stacks of coins and groups of such cells may give the appearance of agglutination. A drop of 0.85 percent sodium chloride will cause dispersion of these cell groups but will not effect true agglutination. Always add a drop of saline and mix before reading slide tests which employ whole blood.
- (7) If hemolysis occurs, a false negative report may be given since no agglutination is evident. A check with the microscope will show that the cells have been hemolyzed. Partial hemolysis is more difficult to recognize since agglutination may be inhibited. Hemolysis has the same significance as agglutination (*b.* (7) above). It may be avoided as a source of error by inactivating the sera at 56° C. for 20 to 30 minutes before setting up either the tube or slide test. Hemolysis will then be prevented and agglutination can be recognized.
- (8) If fresh serum is mixed with citrated whole blood, clotting may occur on the slide and the test is invalid. This is due to an excess of thrombin in fresh serum. Thrombin is destroyed at 56° C. for 15 to 30 minutes. This difficulty may also be obviated by using plasma instead of serum.

d. **AGGLUTINATION.** Occasionally the patient's serum is found to agglutinate the cells of several donors even though of the same blood group. Also the patient's cells may agglutinate in the sera of donors of the same group. Under these circumstances, test the patient's cells in his own serum and if agglutination occurs the presence of auto-agglutinins is indicated. Usually these are only active in the cold and disappear at 37° C. Wash the patient's cells in 0.85 percent sodium chloride and make a fresh 2 percent suspension and repeat the cross-matching at 37° C. If no agglutination occurs, the donor is satisfactory. The transfusion must be carried out with caution and the patient must be carefully observed and the transfusion stopped at any sign of reaction.

e. **RH.** All donors and recipients should be Rh typed as well as A-B-O grouped and it is desirable that Rh negative recipients receive only Rh negative blood. It is essential that Rh negative women who have not passed the child-bearing age receive Rh negative blood and Rh negative men and women who may receive multiple transfusions should receive Rh negative blood. (See par. 100.) It is absolutely essential that Rh negative patients who have developed anti-Rh agglutinins receive only Rh negative blood.

92. Selection of Universal Donors

a. Donors belonging to group O are often used as universal donors because their cells are not ordinarily agglutinated by the serum of any of the other three groups. Rarely, however, group O persons are encountered with such potent isogglutinins that the dilution of their serum in the patient's circulation may not suffice to prevent a hemolytic reaction. Preferably only those group O persons should be used as universal donors whose serums have been shown not to have excessively high titers of isoagglutinins by actual titration. (See *b* below.) In an emergency, any donor certified as belonging to group O, as proved by complete tests on cells and plasma, may be used provided that the blood is transfused slowly.

b. The test to establish the acceptability of a universal donor is as follows:

- (1) Prepare a 1:50 solution of plasma by adding 0.1 ml of plasma to 4.9 ml of saline solution.
- (2) Place 1 drop of the diluted plasma on a slide and add 1 drop of the patient's cell suspension. Mix well with a wooden applicator or toothpick and observe the mixture for about 10 minutes, tilting the slide about once a minute.
- (3) If no or only weak agglutination occurs within 10 min-

utes, the titer of incompatible agglutinins is not excessively high and the donor may therefore be used. If agglutination visible to the naked eye occurs, the donor should not be used. It is well to mention that group O patients who have previously received transfusions of pooled plasma not infrequently develop a dangerously high isoagglutinin concentration.

c. The use of a universal donor does not obviate the need for cross-matching tests (par. 91), although, in an emergency, these tests may be omitted if certified group O blood is used. This procedure is particularly safe if the donor is known to have weak isoagglutinins in the plasma and is Rh—. (See par. 97.)

93. Sources of Error in Grouping

a. GENERAL. Fresh plasma may rarely give hemolysis instead of agglutination, especially in warm climates. Hemolysis is more frequently observed when fresh serum is employed. Care should be taken not to read this as a negative reaction. Another source of confusion, namely, pseudoagglutination, is discussed in *b* below.

b. PSEUDOAGGLUTINATION. In many patients with rapid sedimentation rates due to severe sepsis or other causes, rouleaux formation may be confused with true agglutination when grouping the patient's plasma with known cells or when cross-matching with the donor's blood cells. Rouleaux formation can often be recognized under the high-dry power of the microscope by the appearance of loose clumps of red cells with their flat surfaces in contact to resemble stacks of coins. Pseudoagglutination should be suspected whenever unexpected clumping is encountered and is almost certainly present if the patient's cells suspended in his own plasma show a similar phenomenon. However, a rouleaux formation is usually broken up by stirring, a procedure that, as a rule, intensifies true agglutination.

94. Preparation of Grouping Serums

a. GENERAL.

- (1) Occasionally an emergency arises when grouping serums are badly needed and none are at hand. Strong B serum is difficult to obtain because of the scarcity of group B donors in general. It is wise, therefore, always to have an up-to-date list of donors among the local detachment, with a note as to the strength of the reaction in each case.
- (2) Choose a person of the desired group whose serum is known to have potent isohemagglutinin (par. 95) and

take the blood by venipuncture. Enough serum can be obtained from 30 to 50 ml of blood to last over almost any emergency. Serum that must be used immediately or within 1 to 7 days after collection should be inactivated by heating in a water bath at 56° C. for 30 minutes, to avoid hemolysis which may mask agglutination. Reasonable care should be taken to maintain asepsis while drawing the blood and separating the serum, because sterile serum will retain its full strength for a long time.

b. COLLECTION OF BLOOD. It has been found best to collect the blood in sterile stoppered centrifuge tubes or bottles. Allow the blood to clot and then shake the containers gently but sufficiently to break up the clot so that the greatest possible yield of serum can be obtained.

c. SEPARATION OF SERUM. Centrifuge the containers of blood at 1,500 to 2,000 rpm to separate the serum from the broken clot, or allow the serum to separate in the refrigerator overnight. Decant or pipette off the clear serum into sterile containers, then recentrifuge, if necessary, to get rid of the remaining red cells, and decant into sterile containers.

d. PRESERVATION OF SERUM. When possible it is desirable to add chemical preservatives to the serums, although in emergencies they may be kept for some time without preservatives, especially in the cold. (See (3) below.) The addition of dyes and a preservative solution to the serums serves both to minimize bacterial growth and to facilitate their identification.

(1) *Preserving and coloring group A serum.*

- (a) Have ready a 1 percent aqueous solution of neutral acriflavin and a 1 percent aqueous solution of phenylmercuric nitrate or borate or Merthiolate.
- (b) To each milliliter of clear group A serum add 0.015 ml of acriflavin solution and 0.01 ml of preservative solution.
- (c) Mix thoroughly, store in 2- or 5-ml sterile vials sealed with rubber stoppers, and keep in the refrigerator when not in use.

(2) *Preserving and coloring group B serum.*

- (a) Have ready a 1 percent aqueous solution of brilliant green.
- (b) To each milliliter of clear group B serum add 0.01 ml of the brilliant green solution, and 0.01 ml of the preservative solution.
- (c) Mix and store as for the group A serum.

- (3) If long preservation is desired, store the serums in the frozen state in small quantities, for example, 1 to 5 ml. The frozen serums are thawed as needed.

95. Criteria for Selection of Potent Grouping Serums

a. GENERAL. The criteria for the selection of potent grouping serums depend on biologic reactions and are consequently subject to considerable variation. General methods are employed in various laboratories for selecting grouping serums. All these methods are subject to variations in sensitivity of test cells, probable variations in properties of the agglutinins in the serum, the protein concentration employed, the intrinsic stability of the preparations, and probably other factors still unknown.

b. METHODS.

(1) *Group A serum (anti-B).*

(a) *Minimal titer.* Prepare a 1:16 *dilution* of the serum by mixing 0.1 ml of serum with 1.5 ml of saline solution. Mix one drop (0.05 ml) of the diluted serum on a slide at room temperature with one drop (0.05 ml) of a group B fresh cell suspension prepared as directed in par. 87 d. If possible, set up a parallel test with a cell suspension from a second person of group B. Mix with an applicator or toothpick, agitate by rocking the slide to and fro at intervals of 1 minute. The titer of the serum is satisfactory if agglutination readily visible to the naked eye appears in less than 10 minutes with both bloods.

(b) *Speed and intensity of agglutination (avidity).* Set up a test similar to that described in (a) above, using *undiluted serum* and rock the slide continuously. Agglutination must be visible to the naked eye within 15 seconds, and agglutination should be complete within 30 seconds.

(c) *Specificity.* It is recommended that the serum be used to test at least 50 bloods taken at random, in parallel with a known serum, with satisfactory results, before being considered acceptable.

(2) *Group B serum (anti-A).* The test is performed much as for group A serum, except that account must be taken of weakly reacting A agglutinogens (A_2 and A_2B).

(a) *Minimal titer.* Test as described for group A serum. The 1:16 dilution should agglutinate A_2 cells within 10 minutes.

- (b) *Speed and intensity of reaction (avidity).* Test as described for group A serum against two suspensions of cells, at least one of them sub-group A_2 . Distinct clumping should be visible within 60 seconds with the A_2 cells, and within 15 seconds with the A_1 cells.
- (c) *Specificity.* Carry out tests similarly to above with 50 bloods taken at random, and include, if possible, at least one blood of subgroup A_2B . Agglutination of A_2B cells is more readily recognized if a 4 percent suspension of cells is used.

96. Identification of A_1 , A_2 , A_1B and A_2B Bloods

a. The simplest method is to use a commercial, absorbed B serum (anti- A_1), if obtainable. This agglutinates bloods of subgroups A_1 and A_1B , but not those of subgroups A_2 and A_2B . The next best procedure is to test a series of known group A and group AB bloods with one or two weak group B serums. Usually some of the bloods are definitely more weakly agglutinated than others. These weakly reacting cells are used as A_2 (or A_2B) in the above tests.

b. Recognition of these subgroups is mentioned here only for the purpose of selecting grouping serums. Mutual isoagglutination among these subgroups is not regarded as a cause of hemolytic reactions.

97. Rh Factor

The red blood cells of approximately 87 percent of human subjects, irrespective of their blood groups, contain one or more of the agglutinogens collectively designated as "Rh," related to a similar agglutinin found in the red cells of rhesus monkeys. Apparently there are no naturally occurring anti-Rh agglutinins, but the Rh— individuals (13 percent of the population) are capable of forming anti-Rh agglutinins. This may occur when repeated transfusions of Rh+ blood are given to an Rh— subject or when an Rh— woman bears an Rh+ fetus from an Rh+ father. Not all Rh— persons develop demonstrable isoagglutinins under these circumstances, but following such isoimmunization, when it occurs, the Rh— individual may suffer severe hemolytic reactions when transfused with Rh+ cells. Manifestations of Rh isoimmunization usually do not appear until after several transfusions (a variable number), when reactions, usually mild at first, may occur and increase in severity following each succeeding transfusion with Rh+ cells. Manifestations of Rh isoimmunization usually do not occur

in first pregnancy of the Rh— woman, but in pregnancies that occur after isoimmunization is initiated, the anti-Rh agglutinins increasingly formed by the mother may cause in her Rh+ infants a disease known as erythroblastosis fetalis. This disease is characterized by hemolytic anemia with numerous erythroblasts in the blood.

98. Application

In a patient receiving repeated blood transfusions who has had reactions to previous transfusions, it is important to rule out Rh isoimmunization as a cause of the reactions by Rh typing of the recipient and donor. Should any woman with a history of having born an erythroblastotic infant require a transfusion, Rh typing of recipient and donor should be done before any transfusion, because in such cases even a first transfusion may provoke a fatal reaction. In such cases Rh— blood of a compatible regular group can be safely given.

99. Potent Anti-Rh Testing Serums

These are obtained most readily from the blood of mothers who have borne babies with erythroblastosis fetalis. However, only about 2 percent of these mothers yield serum that has a sufficiently high titer for testing purposes, and the Rh specificity is not always the same. Before distribution for use the testing serum should have had its anti-A and anti-B agglutinins (if present) neutralized by the addition of group specific substances.

100. Rh Typing

a. Rh typing is performed with anti-Rh₀ "slide" serum furnished by the Army Medical Service Graduate School. It will agglutinate 85 percent of blood samples of white persons.

b. This serum is prepared from the serum of sensitized persons. It has been modified by the addition of protein to increase its avidity and the anti-A and anti-B agglutinins have been neutralized. It contains the blocking or incomplete antibody and consequently reacts only when cells are suspended in serum, albumin, or plasma. It does not contain the saline agglutinating antibody and for this reason, SALINE CELL SUSPENSIONS SHOULD NEVER BE USED WITH EITHER OF THESE TESTS.

c. The serum should be kept in the refrigerator when not in use. For dependable results all the serum except that to be used for that day's testing should be kept frozen in the freezing compartment of the laboratory refrigerator. If only a few tests are performed

at one time, it may be advisable to divide the serum into several portions and store in separate sterile bottles. Stopper all bottles or tubes even if they are frozen to prevent evaporation.

d. Draw 5 ml of blood in a tube containing oxalate (par. 70c), mix and transfer 1.0 ml to a second tube. Centrifuge the blood remaining in the first tube and separate the plasma, placing 1 ml of plasma in the third tube to which is then added 1 drop of the whole blood from the second tube. This gives a 2 percent suspension of cells in their own plasma in the third tube. Blood from the second tube (whole blood) is used for the slide test and blood from the third tube (2 percent suspension) is used for the tube test. If it is desired to determine blood groups O, A, B, and AB on this specimen, place 1 drop of the whole oxalated blood in 1 ml of 0.85 percent NaCl and proceed as in par. 88.

e. If only clotted blood is available, prepare a 2 percent suspension of cells in their own serum or in 20 percent bovine albumin. This may be done by decanting the serum from the clot and shaking the remaining clot and serum to loosen some cells. A drop of this suspension of cells in 1 ml of serum or 20 percent bovine albumin will give approximately a 2 percent suspension. Twenty percent bovine albumin is made by adding 10 ml of sterile 0.85 percent saline solution to the 30 percent bovine albumin in the vial.

f. If specimens are not fresh, it is preferable to wash the cells in physiological saline solution by adding several drops of blood to 2 or 3 ml of saline solution, mixing, centrifuging, and decanting the saline. One drop of packed, washed cells in 2 ml of serum, plasma or 20 percent bovine albumin will give approximately a 2 percent suspension.

g. The tube test is as follows:

- (1) In a small test tube, mix 1 drop of the 2 percent suspension of cells and 1 drop of the anti-Rh₀ serum.
- (2) Centrifuge immediately for 1 to 2 minutes at 500 to 1000 rpm.
- (3) With very gentle agitation, shake the cells loose in the tube and read over a mirror, or a light covered with ground glass, or over a white background.
- (4) If there is agglutination, the blood is Rh positive. If there is no agglutination, the blood is Rh negative.
- (5) Sources of error:
 - (a) Too little serum. No more than 1 drop of cell suspension should be used, but 1 to 2 drops of anti-Rh₀ serum may be used.

- (b) Too rough handling breaks up the reaction. Do not shake the tubes, merely roll or twist the tube.
 - (c) Unfamiliarity with negative reactions.
- h. The slide test is as follows:
- (1) On a warm (37° C.) glass slide place 1 small drop of anti-Rh₀ slide serum and 1 large drop of whole, oxalated blood or a 40 to 50 percent suspension of cells in their own serum or in 20 percent bovine albumin.
 - (2) Mix the 2 drops together in an area the size of a quarter and then slowly rock the slide back and forth and around. Observe over a mirror, or over a light covered with ground glass, or over a white background.
 - (3) If the blood is Rh positive, agglutination will occur in 30 to 60 seconds and be complete in three minutes. All tests should be discarded at three minutes as drying may lead to false positive readings. Agglutination will be unmistakable, although it will not have the granular appearance of the A and B agglutination. At the end of the three minutes, add 1 or 2 drops of physiological saline solution in order to avoid confusion because of rouleaux formation. If the blood is Rh negative, there will be no agglutination. All negative and doubtful tests must be checked by the test tube technic described above.
 - (4) It is desirable to set up controls of known Rh positive and known Rh negative cells with the test serums until the technician is fully acquainted with the normal reactions of the test.
 - (5) Sources of error.
 - (a) *A dilute cell suspension.* It is important to use either whole oxalated blood or a 50 percent suspension of cells in serum or plasma or in 20 percent bovine albumin. It must be remembered that, in very anemic patients, whole blood is *not* a 50 percent suspension but may be as low as a 10 or 20 percent suspension. In such cases a 50 percent suspension may be obtained by centrifuging the blood, removing enough plasma to obtain a 50 percent dilution, and resuspending the cells.
 - (b) *Too much anti-Rh₀ serum.* The test works best when the drop of anti-Rh₀ serum is the same size or smaller than the drop of blood.
 - (c) *Unfamiliarity with negative reactions.* Set up controls of Rh positive and Rh negative cells until familiarity with the test is assured.
 - (d) *The use of cold reagents.* The test works best at 37°

C. Slides should be warmed on a substage lamp, or on a heated viewing box. Tests may be performed on the flat surface of a bottle filled with water and kept in the incubator.

i. The following precautions should be observed in conducting the tube test and slide test:

- (1) *Blood must be fresh.* Clotted or oxalated blood may be used up to 5 days (although it is safer not to do so), but cell suspensions must be used within a few hours — no longer than the same day prepared.
- (2) Strict adherence to the conditions of the test must be observed.
- (3) Thorough familiarity with the appearance of positive and negative Rh agglutinations is essential. It is strongly recommended that all blood samples be tested with both the test tube and the slide method until the technician is thoroughly acquainted with both. There are very important differences in the slide and the test tube technics. **BLOODS FOUND TO BE RH NEGATIVE WITH THE SLIDE METHOD MUST BE TESTED IN THE TEST TUBE.**

101. Selection of Rh Negative Donors

The Rh typing serum distributed by the Army Medical Service Graduate School agglutinates only cells with the Rh_0 antigen, Rh_0 (D), Rh_0' (CD), Rh_0'' (DE), and $Rh_0' Rh_0''$ (CDE) and does not agglutinate rh' (C), rh'' (E) or $rh'rh''$ (CE) cells. These latter cells will be typed Rh negative and if given to an individual who has become sensitized to one of these antigens, a serious reaction may develop. To avoid such a possibility, it is important that the method of cross-matching be one that will reveal Rh incompatibilities (par. 91). A further check may be made by subtyping Rh negative donors with anti-serums effective against rh' and rh'' . Such serums are not supplied through medical supply channels at the present time but may be purchased commercially. Anti- Rh_0' and anti- Rh_0'' serums are more readily available than anti- rh' and anti- rh'' serums and are in general use. Rh negative cells (no agglutination with anti- Rh_0 serum) which agglutinate with anti- Rh_0' serum and not with anti- Rh_0'' serum are rh' and those which agglutinate with anti- Rh_0'' serum and not with anti- Rh_0' serum are rh'' and cells agglutinated by both anti- Rh_0' and anti- Rh_0'' are $rh'rh''$. In selecting Rh negative donors only those whose cells fail to agglutinate with all three anti-serums (anti- Rh_0 , anti- Rh_0' and anti- Rh_0'') should be used.

CHAPTER 3

GENERAL CHEMICAL TECHNIC

Section I. TYPES OF CHEMICAL ANALYSES

102. Qualitative and Quantitative Analyses

Qualitative analyses are performed on specimens to learn whether certain substances are present or absent. Quantitative analyses measure the amounts of some or all of the constituents present. The *detection* of sugar or albumin in the urine is a qualitative analysis. The *determination* of the *amount* of sugar or albumin that is present is a quantitative analysis.

103. Types of Quantitative Analyses

Quantitative analyses may be made in many ways. The methods most frequently employed are gravimetric, volumetric, colorimetric, photometric, and gasometric.

a. In gravimetric analysis the constituent sought is separated from solution as an insoluble compound of known chemical composition, or is made to react and produce such a compound, which is then dried or ignited, and weighed. From the weight of this compound the weight of the desired constituent is computed.

b. In volumetric, or titrimetric, analysis the compound sought is not weighed, but is determined by measuring the volume of a solution of known concentration that reacts with it.

c. In a colorimetric method the substance to be measured is either itself colored or is made to react with other chemical compounds to produce a colored substance. The concentration of the substance is estimated from the intensity of the color by matching against a color standard representing the substance in known concentration.

d. In a photometric method the concentration of a substance in solution or suspension is estimated from the percentage of light of specified wave length that is absorbed in passing through a known depth of the solution. At present, analytical photometry is most frequently applied to solutions in which the estimated substance is a colored solute, but it can also be applied to solutions in which the rays absorbed are ultraviolet or infrared, and to suspensions.

e. In a gasometric method the substance sought is either measured as a gas, or is made to react and produce a gas to be measured. To estimate the amount of gas, one may either bring the

gas to atmospheric pressure and note the volume occupied, or may bring it to a definite volume and measure with a manometer the pressure which it exerts. From either the volumetric or the manometric measurement, together with the temperature, the weight of gas can be calculated. Although not an item of standard equipment in Army laboratories, the manometric apparatus of Van Slyke and Neill is used in many of the larger Army laboratories; it serves to determine the blood gases, and also other constituents, such as urea, sugar, calcium, fats, amino acids, and Kjeldahl nitrogen.

Section II. THE ANALYTICAL BALANCE

104. Range and Use

a. RANGE AND USES OF STANDARD ANALYTICAL BALANCE AND MICROBALANCE. The *standard analytical balance* has a capacity of 100 to 200 gm and is capable of reproducing weighings within 0.1 mg when loaded. It is used for gravimetric analyses, for the preparation of primary standard solutions, and for other precise weighings. Precision to 0.1 mg requires the use of standardized weights, and correction for their deviations from their marked values. Weights can be obtained with certificates of their accuracy specified within certain tolerances or by corrections. Such certificates are provided by the Bureau of Standards. Cheaper weights can be standardized by comparison with a set of Bureau of Standards weights or by methods described in text books. The rider must also be of tested accuracy. In "chainomatic" balances the weights up to 100 mg are replaced by a chain. It is imperative that the chain be tested at 10 mg intervals by comparison with standardized weights.

b. With the introduction of microchemical methods there is an increasing use of the *microbalance*, which permits weighing several grams with a reproducibility of about 0.003 mg. With this balance, samples of 4 or 5 mg suffice for accurate analysis. The special points of technic in its use will not be discussed here, as it is not in general use in the Army. For details, see Niederl's book on microanalysis cited in the appendix.

105. General Construction and Principle of Balance

The critically important parts of an analytical balance are the beam and knife edges. The beam is a horizontal lever the two arms of which, as measured from central to end knife edges, should be exactly equal. At the center is a downward pointed knife edge

which rests on a plate of agate or steel when a weighing is made. Equidistant from the central knife edge and exactly parallel thereto are two upward pointed knife edges that support the plates of agate or steel from which are suspended the stirrups and pans. The three knife edges should lie in the same plane. A device, called the arrest, controlled by a knob outside the balance case, serves to bring all supports out of contact with the knife edges when the beam is not in use. A pointer, on which there usually is a movable weight, and which swings over a fixed scale, is attached to the beam to show its position. At one or both ends of the beam there may be small adjustable nuts the movement of which serves to equalize the effective weights on the two sides. The center of gravity of the beam should lie slightly below its support; if it were above its support the beam would be unstable. When the freely moving system comes to rest the pointer will be at the "rest-point." If now a small weight is placed upon one pan, that side of the beam will tip down, and if the system is again allowed to come to rest the pointer will be at a new position determined by the weight of the beam, the length of the arms, the weight added to the pan, and the distance between the center of gravity and the middle knife edge which serves as the point of support. The center of gravity may be raised by raising the weight attached to the pointer arm. This change will increase the sensitivity of the balance. (See par. 106.) A plumb bob, or spirit level, serves to level the instrument so that the plane in which the knife edges lie will be horizontal. A case protects the instrument from dust, sudden changes in temperature, and drafts of air.

106. Sensitivity of Balance

a. MEASUREMENT OF DEFLECTION SUM. The sensitivity of a balance may be expressed by the number of divisions on the pointer scale that the rest-point of the pointer is shifted by a weight of 1 mg. In practice, however, the sensitivity is more commonly expressed by the change in *deflection sum* caused by 1 mg. The *deflection sum* is the *algebraic sum of the average of the right swings and the average of the left swings of the pointer*, measured from the zero point on the scale, *the swings to the right being assigned plus values and the left swings minus values*. One mg changes the deflection sum twice as much as it changes the rest-point. For example, assume that when no loads are placed on the pans, the rest-point as indicated by the pointer is at the zero point of the scale. Now, if adding 1 mg to the right pan causes the pointer to begin oscillating between 0 and -5 on the scale the deflection is -5, but the point of final rest will be -2.5. In con-

temporary usage the sensitivity of this balance would be expressed as 5, rather than 2.5. If the ends of the beam are lower than the middle, or if the beam bends significantly when loaded, the sensitivity will decrease when the loads increase. The sensitivity of the balance should be determined with loads covering the range of its use, as between 0 and 100 grams. If the sensitivity varies with the load, make a table or graph showing sensitivity versus load, for use in future weighings.

b. **EXAMPLE.** The following example illustrates a *determination of the sensitivity* with a load of 10 grams, and the *technic of measuring the deflection sum*: A 10-gram weight is placed on each pan, and the swings of the pointer are counted. The first 2 or 3 swings are let pass before the counting is begun, as the first swings may not be regular. Then an odd number of swings is counted: +4.2, -2.5, +4.0, -2.3, +3.8. The average of right swings is +4.0 and of left swings -2.4. The deflection sum is therefore +1.6. The rider is now placed at 1 mg and the swings are again counted: +1.2, -4.5, +1.0, -4.3, +0.8. The deflection sum is now -3.4. One mg has changed the deflection sum from +1.6 to -3.4, or 5.0 divisions. The sensitivity is therefore 5.0 divisions when the balance has a 10-gram load.

In measuring the deflection sum, *an odd number of total swings is used* (for example, 3 to the right and 2 to the left, as in the above example) in order to avoid error from the decrement in amplitude which occurs with each swing. By raising or lowering the movable weight on the pointer the sensitivity can be altered. A deflection sum of 5 divisions for 1 mg is a convenient sensitivity.

107. Checking Zero Point of Balance

The zero point of the balance is the deflection sum with empty pans. It may not be zero on the scale, and it may change during the course of a day. If the weight of substance is measured by the difference between two weighings made one immediately after another, as when a sample of material is weighed on a counter-balanced watch glass, one may neglect correction to the zero point. It will be the same in both weighings, and the difference between them will not be affected. If, however, one wishes to determine the weight of an object in a single weighing, one must determine the zero point of the balance when no load is on the pans, and algebraically subtract its equivalent in milligrams from the uncorrected observed weight. For example, if the sensitivity is 5 and the deflection sum with pans empty is -2.0, add 0.4 mg (subtracting -0.4 mg) to the uncorrected weight. If a crucible is weighed empty in the morning, and with a precipitate in the

afternoon, the zero point should be checked with each weighing, and the corrections used if they are different.

108. Methods of Weighing

a. **WEIGHING WITH RIDER ADJUSTED BY REPEATED TRIAL.** Place the object to be weighed on the left pan. With the forceps, place on the right pan a weight that is judged to approximate the weight of the object. Gently lower the beam a little and note which way the pointer moves. Change the weights systematically, lowering the beam after each change until the weights outweigh the object less than 1 gm. Then remove 1 gm from the weight pan, and add fractional gram weights until the range covered by the rider is reached. Close the balance case and adjust the rider until the pointer swings equal distances to the right and left of zero. A swing of 5, or less, scale divisions to right and left of the mean is preferable to a wider swing. Record the weight from the empty places in the box and check by counting the weights on the pan. Recheck the weights as they are placed in the box.

b. **WEIGHING BY SWINGS.**

- (1) This method saves the time taken to shift the rider by trial over the last fraction of a mg. It also enables one to estimate fractions less than 0.1 mg, but such estimations are valid with a standard balance only if unusual precautions with regard to calibrated weights, rechecking of the zero point, etc. have been taken.
- (2) The object is balanced as described above to within 1 mg by weights and rider. The deflection sum is then noted, as described in paragraph 106, and is divided by the previously determined sensitivity of the balance. The quotient, deflection sum divided by sensitivity, is the fraction of a mg to add to or subtract from the weight indicated by the position of the rider. For example, if in weighing a crucible the weights and rider indicate 12.436 gm, the deflection sum is —1.4 scale divisions, and the sensitivity of the balance with a 12-gm load is 5.0 divisions, one divides 1.4 by 5.0 and subtracts the quotient, 0.28 mg (or 0.3 mg) from 12.436 gm, to obtain 12.4357 gm as the weight of the crucible (without correction for the zero point of the balance). The 0.3 mg in this case is subtracted because the deflection sum, —1.4 divisions, was negative to the left. The calculation from the deflection sum may be checked by placing the rider on the calculated point and noting whether the deflection sum is then zero.

c. CORRECTION FOR ZERO POINT. After weighing by either method in *a* or *b* above, correct for the zero point as described in paragraph 107, if the correction is necessary.

109. Electrical Effects

If the object to be weighed is of glass or porcelain and has recently been wiped with a cloth, it is allowed to stand for several minutes before weighing, and when the weighing is finished the object is left on the pans for several minutes more and is reweighed to make certain that its weight has attained constancy. Wiping a glass or porcelain object is likely to give it a charge of static electricity that may take $\frac{1}{2}$ hour or longer to disappear. The effect lasts longer when the air is dry, as on a cold winter day, than when it is humid. A Pyrex glass vessel may require several hours on a dry day to reach constant weight. Static electricity does not affect metal objects, and when possible it is desirable to make weighings in metal rather than porcelain or glass containers.

110. Temperature Effects

The object weighed must be in temperature equilibrium with the atmosphere of the balance, otherwise convection currents in the air above the pan will be set in motion and will affect the weighing. Also, glass and porcelain objects under ordinary atmospheric conditions absorb a slight film of moisture, which varies with the temperature.

Weighing is easiest when the object can be allowed to come to constant weight in the room air before weighing. When a porcelain crucible has been ignited with a substance, like barium sulfate, that does not absorb moisture from the air, the crucible is left to cool $\frac{1}{2}$ hour in the open air, then is left 10 or 15 minutes near the balance case, and is weighed. A platinum vessel cools more rapidly. If, however, the ignited or dried substance is one that gains weight by absorbing atmospheric moisture, the crucible is allowed to cool in the open air only partly, and while still warm is placed in a desiccator for over $\frac{1}{2}$ hour. Before removing the crucible from the desiccator the approximate weights to balance it are placed on the weight pan, and the weighing is then made as quickly as possible.

Regardless of how a material is prepared for weighing, the crucible or other vessel must be prepared in the same manner both for the preliminary weighing when empty and for the final weighing when it contains the material.

In weighings with a microbalance the effects of both temperature and static electricity require special attention to avoid gross errors.

III. Precautions in Using an Analytical Balance

a. The balance should rest on a firm support that is as free as possible from vibrations. These can interfere with weighings and shorten the life of a balance, especially a delicate microbalance. If a base free from vibration is not available, the feet of the balance can be set on wide cork stoppers about 2 cm high. More elaborate antivibration bases are described by Niederl. (See the app.) Direct sunlight or intense and unevenly distributed artificial light should not fall on the balance.

b. If the knife edges were in constant contact with the agate plates they would be worn by the continuous vibrations of the building. Therefore, the arrest should be adjusted to hold the supports out of contact with the knife edges when the balance is not in use. Frequently, separate arrests for the pans are provided. If the beam is not in arrest and a heavy object is put on one of the pans, a delicate knife edge may be injured. To avoid this, make a routine practice of inspecting the balance each time before using it to make certain that the beam and pans are in arrest.

c. Final rider adjustments should be made with the balance case closed to prevent errors from air currents. When not in use the case should be kept closed to exclude dust.

d. Chemicals should not be placed directly on the pans, but in watch glasses or other containers. Counterbalanced scoops made from sheet aluminum or copper are convenient, and prevent error from static electricity.

e. Never handle the weights except with the forceps.

f. Keep the pans and the balance floor clean by daily use of a camel's hair brush.

g. In placing crucibles, flasks, and other objects on the pans, always use forceps or tongs to handle them. In general, to avoid touching and dislocating parts of the balance, the hands are never put inside the balance case farther than is necessary in handling forceps and balance brushes.

h. When loads are exactly balanced a slight force may be required to set the beam in motion. This is usually supplied by gentle lowering of the beam from supports that are seldom exactly equal. If the beam fails to swing, it is started by lifting the rider for a moment, or by a current of air blown against a pan from a medicine dropper with a curved tip. *Never start* motion by touching a pan or by rough lowering of the beam.

Section III. VOLUMETRIC GLASSWARE

112. Volumetric Flasks

a. GENERAL. Sizes needed are 1 liter, and 500, 250, 100, 50, 25, and 10 ml. Most flasks are calibrated to *contain* a definite volume, and only one mark is etched on the neck. Some, however, have two marks, the lower indicating that the vessel contains, and the upper that the vessel delivers, the specified volume of solution. The calibration by a responsible manufacturer may be accepted as accurate enough for most flasks, particularly those of 100 ml and greater capacity. But for assured accuracy it is necessary to check the marks by recalibrating the flasks.

b. CALIBRATION OF FLASKS. The following procedure may be used to calibrate flasks to *contain* specified volumes of liquid. Weigh the dry flasks on a balance of appropriate size and sensitivity, or, instead of weighing the empty flask, it may be counterpoised with shot. The water to be introduced should be weighed to 1 part in 1,000; that is, a 10 ml flask must be weighed to 0.01 gm and a liter flask to 1 gm. Calculate from table VII the weight

*Table VII. Apparent Weights and Volumes of Water Weighed in Air for Use in Calibration of Volumetric Apparatus**

Temperature	Weight of 1 ml	Volume of 1 gm	Temperature	Weight of 1 ml	Volume of 1 gm
C°	gm	ml	C°	gm	ml
10.....	0.9983	1.0017	25.....	0.9981	1.0039
11.....	0.9983	1.0017	26.....	0.9959	1.0041
12.....	0.9982	1.0018	27.....	0.9956	1.0044
13.....	0.9981	1.0019	28.....	0.9954	1.0046
14.....	0.9980	1.0020	29.....	0.9951	1.0049
15.....	0.9979	1.0021	30.....	0.9948	1.0052
16.....	0.9978	1.0022	31.....	0.9945	1.0055
17.....	0.9977	1.0023	32.....	0.9942	1.0058
18.....	0.9975	1.0025	33.....	0.9939	1.0061
19.....	0.9973	1.0027	34.....	0.9936	1.0064
20.....	0.9972	1.0028	35.....	0.9933	1.0067
21.....	0.9970	1.0030	36.....	0.9929	1.0071
22.....	0.9968	1.0032	37.....	0.9926	1.0074
23.....	0.9966	1.0034	38.....	0.9923	1.0077
24.....	0.9964	1.0036	39.....	0.9919	1.0081
			40.....	0.9916	1.0085

*The figures in this table serve to calculate the weights of water that must be weighed into a glass vessel at the indicated temperatures to fill the vessel to the mark that indicates the designated capacity when the vessel is at 20° C. Corrections are included for the buoyant effects of air on the water and the weights, and for the temperature coefficient of expansion of glass. The weights are for distilled water saturated with air.

of water necessary to fill the flask to the mark at the room temperature, and add weights to this amount to the right balance pan. Fill the flask on the left pan with water until balance is attained, making sure that no drops of water adhere to the neck of the flask. Mark the lowest point of the meniscus with a wax pencil sharpened to a chisel edge, then etch in the mark. An alternative procedure for a flask of 50 ml or less is to deliver into it the specified volume of water from an accurately calibrated delivery pipette. Pay particular attention to the precautions described under paragraph 114 for precise delivery from pipettes. If the manufacturer's mark on a flask is found in error, efface it with crosses etched into the glass.

c. MIXING IN VOLUMETRIC FLASKS. When a solution is prepared in a volumetric flask, the flask is filled to the mark and is inverted at least 10 times in order to insure complete mixing. Each time the flask is inverted it is held in that position until the air bubble entirely leaves the neck. The flask is then returned to the upright position and held there until the bubble has returned to the top of the neck before inverting again.

113. Burettes

a. CARE OF BURETTES.

- (1) After each day's use, burettes, unless permanently attached to solution bottles, are rinsed with water and inverted, with cocks open, to dry.
- (2) No alkaline solution should be left over night in a burette, since it is likely to "freeze" the cock. Furthermore, dilute standard alkaline solutions, 0.1 normal or less, may dissolve enough silicate from the glass to change their titer. The more dilute the alkaline solution the more sensitive it is to change from contact with the glass.
- (3) The glass cocks must be kept clean and lubricated to prevent "freezing" and leakage. Vaseline is a satisfactory lubricant. A minimum of lubricant should be used, and the old removed before lubricating anew.
- (4) As soon as a burette begins to leave droplets, instead of a clean film, when draining from the upper portions, it should be cleaned. It usually will suffice to clean the burette with a burette brush and soapy water, or by filling it with cleaning mixture. The burette then is rinsed repeatedly with tap water, finally with distilled water, and is inverted to dry. If it is needed at once, the

burette is finally rinsed with alcohol and ether and dried by a current of air.

b. TECHNIC FOR FILLING AND ACCURATE DELIVERY OF BURETTES.

(1) *Filling.* Fill the burette to 2 or 3 cm above the zero mark and deliver enough solution to remove air bubbles from the tip. Then run the meniscus slowly down to the zero mark. Wait a minute for drainage from the wall to become complete, then readjust the meniscus to the zero mark if necessary. Remove the droplet from the tip by touching the latter with a glass surface or filter paper. Then proceed at once with the delivery.

(2) *Delivery.*

(a) Delivery from a burette *must not be too rapid* or the film of solution left on the wall will be increased and the burette reading will be too great. For the most accurate delivery the rate of fall of the meniscus should not exceed 5 mm per second. With a 25- or 50-ml burette one may let the solution flow out in rapid drops, but not in an unbroken stream. For a micro-burette (capacity 0.1 to 5.0 ml) one notes with a watch the rate of drop fall that accompanies a meniscus descent of 5 mm per second, and regulates accordingly the rate of drop fall in subsequent deliveries. The smaller the bore of a burette the greater is the ratio of wall surface to volume of solution, and hence the greater the care necessary to obtain uniform drainage in delivery.

(b) As the end point of a titration is approached it may be desirable to deliver the last portions of solutions from the burette in *fractions of a drop*. To do this a little of the solution is permitted to protrude from the tip of the burette, and is detached by touching the tip to the inside of the receiving flask near enough to the solution so that by rotating the solution the droplet is mixed with it.

(c) It is often convenient to attach an *extra tip*, by a piece of fine-bore rubber tubing, to the burette tip. The extra tip is made by drawing out a piece of capillary tubing. One can thus obtain a finer tip, delivering smaller drops, and the extra length enables one, in using the split-drop technic at the end point, to deposit the droplet where it can be easily mixed with the solution in the receiving flask.

(d) When it is desired to deliver drops as small as possible, the burette tip is greased with a thin film of vaseline

which has been mixed with a little caprylic alcohol. This treatment halves the size of the drops delivered; water solutions can thus be delivered in drops as small as 0.01 ml.

c. CALIBRATION OF BURETTES. First clean the burette with chromic cleaning mixture or a soapy brush, and rinse repeatedly with water. Make certain that the cock is perfectly lubricated. Then fill with water at room temperature as described above. For burettes of 10- to 50-ml capacity, deliver the water in portions of 2 ml into a weighing bottle containing a layer of liquid petrolatum about 5 mm thick, the bottle and petrolatum having previously been weighed to 1 mg. After delivering each 2 ml portion, any drop protruding from the tip is removed by touching to the oil surface, and the bottle and its contents are weighed. Multiply the weight in grams of water delivered from the zero mark to each division by the volume of 1 gm, taken from table VII, to calculate the volume delivered. After finishing the calibration, clean and wipe the weighing bottle, put in a fresh layer of petrolatum, and repeat the calibration to check the accuracy of the deliveries. If duplicate results at any point deviate significantly, repeat the deliveries to these points. For each point the correction is calculated as *delivered volume* minus *marked volume*. For example, if at the 8 ml mark the water delivered at 23° C. weighs 8.012 gm, the volume is $8.012 \times 1.0034 = 8.039$ ml, and the correction to the nearest 0.01 ml is +0.04 ml. Similar corrections are calculated at 2 ml intervals for the entire burette. Make a table or, better, a graph of the corrections. Etch a number on the burette, and mark the correction table or curve with the same number. If the burette is of 5-ml capacity, calibrate it at 1 ml. intervals, using a 10-ml weighing bottle.

d. MICROBURETTES.

- (1) Burettes with a capacity of 0.1 to 5 ml, so constructed that the error of delivery does not exceed 0.1 percent of the capacity, are called "microburettes." The construction, internal diameters, outlets, and stopcocks of the smaller microburettes must be quite different from those of standard burettes. The fluid must be delivered in minute drops, and the amount adherent to the tip of the burette must be insignificant. Both objects are attained by using a delivery tip drawn out to a fine point. It is not desirable, however, to make the permanent tip too fine, as it might be too fragile, or be easily clogged with particles, such as bits of grease from the cock. Removable tips of drawn-out, glass capillary tubing can be attached to the permanent tip by heavy-walled, fine-bore

(1 or 2 mm) rubber tubing; or a Luer adapter can be sealed to the burette tip, and then fitted with a small-gauge hypodermic needle (the Shohl burette tip) by which minute drops can be delivered. In the ultramicro-burettes of Rehberg and Scholander, the stopcock is dispensed with, and the flow of liquid is regulated by a micrometer screw working on a mercury reservoir. Such burettes usually deliver with their tips dipping below the surface of the titrated solution.

- (2) There are various devices for calibrating microburettes. One principle, which usually can be applied, is to fill the burette with a concentrated standard solution, and titrate it against a tenfold or hundredfold more dilute solution delivered from a standard burette. For example, the microburette may be filled with 1 *N* sulfuric acid, portions of which are titrated against 0.01 *N* NaOH measured from a standard burette.

c. READING BURETTES.

- (1) The readings are preferably taken at the bottom of the meniscus because this is more sharply defined than the top of the meniscus. Exceptions must be made when solutions are so dark that the bottom of the meniscus can not be seen: such are permanganate and iodine solutions at 0.1 *N* concentration. With such solutions the position at the top of the meniscus is read.
- (2) In making burette readings the most important precaution is to have the *eye at the level of the meniscus*; otherwise error from parallax will occur. The error is easier to avoid if the graduations at the 1- or 0.5-ml intervals are rings going completely around the burette and if the marks at other intervals go halfway around. By sighting across the nearest ring or the ends of the other marks, one can judge whether the eye is at the proper level.
- (3) After recording a reading, repeat the reading; if the drainage of the solution last delivered was not complete at the first reading, the second will be a little higher on the burette. The second reading will not, however, entirely correct error caused by too rapid delivery from the upper parts of the burette.
- (4) For precise readings, especially with burettes of 5 ml capacity, or less, a magnifying glass is useful.
- (5) With a 25-ml burette, duplicate deliveries can be made within 0.01 ml of the mean or correct volume; with a 10-ml burette they can be within 0.005 ml.

114. Pipettes

a. TYPES OF PIPETTES.

- (1) A pipette for measuring a single fixed volume is called "transfer" pipette. One for measuring varying volumes is called a "graduated" or "Mohr" pipette. Graduated pipettes are ordinarily not used for precise measurements, which are made preferably with either burettes or transfer pipettes.
- (2) Most pipettes, except those of 0.1 ml capacity or less, are calibrated "*to deliver*" a definite volume of fluid, without rinsing out the film left adherent on the inner wall. The film is so small a percentage of the total fluid in a large pipette that variations in the volume of the film can be neglected if the delivery is controlled as directed below.
- (3) Pipettes of 0.1 ml capacity or less are usually calibrated "*to contain*" a definite volume. After fluid from such a pipette has been delivered into a receiving vessel the pipette is rinsed with water three times to transfer the fluid adherent to the wall after the first delivery. With very small pipettes the ratio of wall surface to volume content is so great that differences in the manner of delivery or in the physical properties of the fluid can cause relatively large variations in the percentage of fluid left on the inner wall after the first delivery. In hemoglobin determinations on 0.05 ml samples of blood, for example, the use of a "to contain" pipette is essential to accuracy. Sometimes a larger pipette is calibrated with two marks, one "to deliver" and one "to contain."

b. QUICK CLEANING AND DRYING OF PIPETTES.

- (1) *Rinsing with water, alcohol, and ether* (for routine cleaning of numbers of pipettes, see ch. 1). Before a pipette is used to measure a solution it must either be rinsed three times with successive portions of the solution, or must be dry. When the same pipette is used for a series of measurements, and the supply of fluid to be measured is too limited to permit such rinsing, as in most blood analyses, it is necessary to dry the pipette between measurements. Cleaning and drying are accomplished quickly by attaching to one end of the pipette a rubber tube that leads to a bottle connected with a suction pump. The pipette is washed three times or more by dipping the tip into water until suction has filled the pipette; after each filling the end dipped into

the water is lifted and the water is drawn out of the pipette. The pipette is then rinsed once in the same way with 95 percent ethyl alcohol and once with ether. After rinsing with ether, air is drawn through the pipette for a few seconds until it is dry. It is convenient to have permanently at hand three bottles of 150- or 200 ml capacity, bound together and marked "water," "alcohol," and "ether." The bottles are closed by cork, not rubber, stoppers, and the stoppers are marked in the same way as the bottles.

- (2) *Cleaning of greasy pipettes.* When a pipette no longer delivers with clean walls after rinsing with water, alcohol, and ether, it may be cleaned quickly with *hot* chromic-sulfuric acid mixture. The cleaning mixture may be heated and then drawn up into the pipette (*not* by mouth suction, unless a safety tube is interposed); or the pipette may be filled with cleaning solution at room temperature and then warmed by passing the bulb over a small flame or under a stream of hot water. The cleaning mixture is then discharged, and the pipette is rinsed repeatedly with tap water, then with distilled water, alcohol, and ether, as described above.

c. **FILLING A PIPETTE.** In filling a pipette the liquid is first drawn above the mark, and the wet part of the delivery stem is wiped dry. The surplus liquid above the mark is then run out while the pipette is held in a nearly vertical position. As the meniscus approaches the mark the tip of the pipette is touched to a glass surface, or to a filter paper or towel, so that no adherent drop projects beyond the tip when the mark is reached.

d. **DELIVERING FROM A PIPETTE.**

- (1) *Control.* In delivering solutions from all kinds of pipettes, except those provided with stopcocks, the outflow of liquid during part of the process is controlled by pressure of the tip of the finger over the upper opening to regulate the inflow of air. To obtain the necessary control the skin of the fingertip must be neither dry and hard nor wet. A proper condition is obtained by wetting the end of the finger for a moment and then drying it on a towel.
- (2) *Regulating rate of delivery.* For a precise delivery from pipettes the same rule holds as for burettes (par. 113b); the rate of fall of the meniscus must not exceed 5 mm per second. Some pipettes have their outlets so narrow that a sufficiently slow rate of delivery is obtained by letting the liquid flow out freely. A tip with so small a

bore is an inconvenience, however, since it retards filling, and the delivery from the lower stem is unnecessarily slow. It is more convenient to use a pipette having a tip with a moderately wide bore, and to regulate the outflow rate by controlling the inflow of air with the fingertip. In this way a uniform rate of delivery from the entire length of the pipette can be obtained, and maximal precision can be maintained without undue loss of time in either filling or delivery. If less than maximal precision suffices, the outflow can be accelerated.

- (3) *Drainage delivery.* This type of delivery may be used with pipettes of 5 ml capacity or more. When all the liquid that will run out by gravity has been discharged, the tip of the pipette is touched to the glass of the receiving vessel, and is left there for 5 seconds, with the pipette in a nearly vertical position, while drainage is completed. The pipette is then withdrawn *without discharging the drop from the tip.*
- (4) *Blow-out delivery.* This type of delivery may be used with pipettes of any size, and, for precise results, is always used with small pipettes, 3 ml or less. The delivery is performed exactly as the drainage delivery, except that at the end of the 5-second period of drainage the drop in the tip is blown into the receiving vessel. The air pressure to expel the drop may be applied with the breath, or by slipping a short piece of rubber tubing over the upper end of the pipette and bending the tubing double, or by closing the upper end of the pipette with the finger of one hand and warming the bulb of the pipette with the palm of the other hand.
- (5) *Wash-out delivery.* This is used only with pipettes calibrated "to contain." First empty the pipette as described for blow-out delivery. Then fill it with water (or other solvent used if the solution is nonaqueous) two or three times and deliver the wash liquid into the receiving vessel.
- (6) It is important that the laboratory have a system which prevents the possibility of mistaking which delivery pipettes are calibrated for drainage and which for blow-out. The simplest system is to have every pipette calibrated for blow-out. Next in simplicity is to have all pipettes of 5 ml capacity or more calibrated for drainage, and all smaller pipettes for delivery. *No possibility should be permitted that a pipette calibrated for drainage delivery will by mistake be used with blow-out technic, or that a blow-out pipette will be delivered by drainage.*

e. CALIBRATION OF PIPETTES WITH WATER.

- (1) *Checking accuracy of a pipette already marked for delivery.* With the precautions outlined in paragraph 114d, deliver distilled water of known temperature from the pipette into a previously weighed weighing bottle and determine the amount of water delivered. The weight delivered is multiplied by the volume of 1 gm of water (table VII) to calculate the volume delivered. If the volume is inaccurate by an amount that is significant for the uses of the pipette, make a new mark with a chisel-edge wax pencil and test it in the same way. When a correct mark has been located, etch it in, and efface the incorrect mark with crosses. *In the calibration the water must be delivered by the same technic (drainage or blow-out delivery) that is to be employed in subsequent use of the pipette.*
 - (a) To avoid loss of water by evaporation during the delivery and weighing, either of two technics may be used. One is to make the delivery into the bottom of a dry weighing bottle capable of holding about five times the volume of the water; with a bottle of this size, and at least twice as tall as wide, no significant evaporation occurs during the delivery. The instant the delivery is completed the stopper is put into place and the bottle and water are weighed. This procedure is preferable for pipettes of less than 2 ml capacity, since it avoids the slight error that might be caused by adherence of oil to the pipette tip in the procedure described below.
 - (b) The alternative procedure is to make the delivery into a weighing bottle containing a layer of oil about 5 mm deep. The delivery is made with the pipette tip almost touching the oil, and at the end of delivery, whether by drainage or blow-out, the drop of water at the tip is detached by touching it to the oil. No cover need be used with the weighing bottle because the oil delays evaporation of the water. Also, one can make several deliveries into the same bottle without emptying and reweighing it. These conveniences make the oil method desirable for pipettes of 2-ml capacity or more.
- (2) *Calibration of unmarked pipettes for delivery.* Two preliminary marks separated by a definite distance, such as 50 mm, are made with a chisel-edged wax pencil, and the water delivered from each is weighed. From the difference the weight of water contained in each millimeter of

length is calculated, and from this is calculated the number of millimeters from either preliminary mark to the correct mark. The latter is located at the calculated level on the stem, is tested by weighing the water delivered from it, and is etched in.

Example. The weights of water delivered at 20° C. from 10-ml pipette with two preliminary marks 50 mm apart are 9.900 and 10.275 gm, respectively. Hence the weight of the column of water between the marks is 0.375 gm, or $0.375 \div 50 = 0.0075$ gm per millimeter of length. The weight of the 10-ml of water at 20° C. is 9.972 gm, or 0.072 gm more than that delivered from the lower preliminary mark. Hence the correct mark is $0.072 \div 0.0075 = 9.6$ mm above the lower mark.

- (3) *Calibration of pipettes to contain.* In a weighing bottle of five or six times the capacity of the pipette place somewhat more than enough water to fill the pipette, and stopper and weigh. Fill the pipette to the mark from the bottle, and immediately close and reweigh the latter.

f. CALIBRATION OF PIPETTES WITH MERCURY.

- (1) Except for extremely small pipettes, mercury is less desirable than water for calibration. The filling to the mark is more difficult to control with mercury, and a correction must be made for the fact that the menisci of mercury and water curve in opposite directions. For pipettes of 0.2 ml or less, mercury has the advantages of high specific weight and inappreciable volatility.
- (2) Mercury is usually used to calibrate pipettes "to contain." The dry and clean pipette is filled to the mark, and the mercury is delivered into a weighing bottle and weighed. Grams of mercury divided by 13.55 indicate the capacity of the pipette in milliliters uncorrected for the meniscus. To this uncorrected volume one must add the correction for the meniscus; 5 cu mm when the bore of the stem at the mark is 3 mm, 2.5 cu mm when the bore is 2 mm, and 0.5 cu mm when the bore is 1 mm. If the bore is less than 1 mm the correction can ordinarily be neglected.
- (3) To calibrate with mercury a pipette "to deliver" water solutions, one must first wet the clean pipette with water, so that a film remains on the inner wall, and deliver the mercury from the wet pipette. The calibration serves for delivery of water by the blow-out technic. The corrections indicated above for the curve of the mercury meniscus are applied.

115. Graduated Cylinders

a. Cylinders are not so accurate as burettes, pipettes, or volumetric flasks. Hence cylinders are, as a rule, used only for measurements in which a 1 percent error is not important. More accurate measurements, however, can be made with a cylinder if it is calibrated, and if after delivery of the contents, it is allowed to drain for 2 minutes, or is rinsed. A cylinder can be quickly calibrated by filling it by repeated deliveries from a calibrated pipette or flask; for example, a 50-ml cylinder can be calibrated at 10-ml intervals by deliveries from a 10-ml pipette. Cylinders are calibrated "to contain," but it drained for 2 minutes the amounts delivered come sufficiently close to the content for measurements of the accuracy usually attempted with cylinders.

b. For each measurement use the size of cylinder that will be most nearly filled by the volume of fluid measured. Preferably use a cylinder that will be more than half filled.

Section IV. FILTERS AND FILTRATION

116. Paper Filters

a. TYPES OF FILTER PAPER.

- (1) To suit the requirements of differing degrees of fineness in precipitates, and of other conditions, various types of filter paper are provided. The desirable qualities are strength, uniform texture, proper porosity, and freedom from substances that interfere with a particular analysis. If a determination of inorganic constituents or an analysis of ash is to be made, an acid-washed, ash-free filter paper is used. If a fine precipitate, like that of barium sulfate or calcium oxalate, is to be filtered, a fine texture is required. If, however, a coarse, flocculent precipitate is to be filtered, a paper of loose texture is used, since one of fine texture would unnecessarily prolong the filtration. Laboratory supply houses and manufacturers give the characteristics of various filter papers in a user's guide indicating the correct papers for various analyses.
- (2) An analysis, particularly of the small amounts of material determined in blood analyses, may suffer gross error from the selection of improper filter paper. Thus, to obtain a protein-free filtrate from serum precipitated with trichloroacetic acid, to be used in a determination of calcium, only the highest grade, most completely *acid-*

washed filter paper can be used; paper that has not been acid-washed contains amounts of calcium that make the analysis meaningless, and even the acid-washed papers must be tested in blank analyses. On the other hand, if a blood filtrate is to be obtained for a nonprotein nitrogen or urea nitrogen determination, an acid-washed paper is not necessary and in fact is more likely to contain ammonia than papers not washed with acid. In many descriptions of methods the authors indicate the types of filter papers to be used. When this is the case another type of filter paper should not be substituted without testing it.

- (3) To test a filter paper for a specific analysis 10 papers may be put through the routine of washing or ashing, and subsequent determination of the substance sought. If a measurable amount is found, one-tenth of it will be the correction for a single paper. In most microanalyses a blank on the reagents is performed and included in the calculation; if a filtration is included in the analytical process, a filtration is also performed in the blank analysis, so that it will include correction for any significant impurity present in the paper.
- (4) For filtering with suction, as with Buchner funnels, hardened paper is used. Besides its strength, it has the advantage that its smooth surface permits one to remove precipitates from it with a minimum admixture of paper fibers.
- (5) When the analysis is to be performed on an aliquot of the filtrate, *pleated* filter papers afford the most convenient and rapid filtration, because they provide the greatest filtering area. The pleated paper is fitted into a funnel large enough so that its sides rise above the paper, and the mixture to be filtered is poured into the pocket formed by the dry paper. Pleated filters should not be used when a precipitate is to be washed.

b. FOLDING FILTER PAPERS.

- (1) *Making 60° cone with two foldings.* Crease the paper across one diameter and then, without opening the paper, make a second fold exactly at right angles to the first one. The paper should be of such size that when it is fitted into the funnel its upper edge is well below the rim of the latter. With an accurate 60° funnel and a well-folded filter paper, the fit of paper to funnel is so accurate that, when the funnel stem is full of water and therefore exerting suction, no air is drawn into the stem,

even after all the solution has filtered out of the space above the paper. The suction is thus maintained for successive washings, which can be carried through in a fraction of the time that would be required with an ineptly fitted filter paper.

(2) *Making 60° cone with multiple foldings.* Although the method just described is the easiest and quickest for folding a paper, considerable time in filtration may be saved by folding the paper so that the liquid passes through it more rapidly. The following method accomplishes this purpose:

- (a) Fold the paper evenly across one diameter.
- (b) Open up the paper and make a second fold at right angles to the first, creasing the paper on the same side.
- (c) Open and turn the paper over, then make a third fold exactly bisecting two of the quarters, creasing on the opposite side from the first two creases.
- (d) Make a fourth fold at right angles to the last one. (The paper is thus divided into eighths, each segment spanning 45°, with two creases on one side and two on the other.)
- (e) Fold again on the same side as the last, dividing two opposite eighths equally.
- (f) Make a final fold at right angles to the previous one, and on the same side.
- (g) In making the folds be sure all creases pass through the same center point, and protect this point with a finger, as the creases are made, to prevent tearing.
- (h) As the paper is picked up it will tend to shape itself into a cone with four equal sections, each having both a triple and a single thickness of paper. Adjust three of the sections, reserving the fourth one to make the paper fit the funnel exactly. This folding gives alternate triple and single thicknesses, with half of the funnel area covered by a single thickness. It makes for rapid filtration.

117. Asbestos Filters

Asbestos for Gooch filters may be prepared as follows: The long-fibered asbestos sold for filtration is cut into pieces 0.5 to 1 cm long, and digested with concentrated hydrochloric acid on the steam bath for 1 hour or more in order to remove soluble impurities. The fibers are poured upon a Buchner funnel and washed with water till the washings no longer give a test for chloride with

silver nitrate. The washed fibers are then mixed with such a volume of water that, when the mixture is shaken up and a Gooch crucible is filled with the suspension and sucked dry, the fibers will form a mat 1 or 2 mm thick. When a crucible is thus prepared with a mat, it is advisable to run about 500 ml of water through in order to detach all fibers that are not firmly knitted into the mat.

To prepare the Gooch crucible for weighing, place it inside a larger porcelain crucible and heat gradually with a burner, the flame of which is turned low and placed at some distance below the crucibles. When no more steam is evolved from the asbestos mat, the heat is increased until the lower crucible begins to show red. The crucibles are then cooled. (For weighing, see par 110.)

118. Porous Glass Filters

These filters are made by fusing a disk of sintered glass in a funnel of proper size. They come in many shapes and sizes, and the fritted glass disks are of numerous grades of porosity, from extremely fine to coarse. In making the disks, powdered glass is sifted to a uniform degree of fineness, pressed into a disk, and heated just enough to cause the individual grains of glass to adhere to each other. Fritted glass filters may be used for filtering anything except solutions of hydrofluoric acid and hot, strong alkali. They are especially useful when filtration by suction is employed.

119. Funnels

a. GLASS FUNNELS FOR QUANTITATIVE WASHING OF PRECIPITATES. These funnels must have an accurate internal angle of 60° , and straight inner walls to permit accurate fitting to folded filter paper. The stem should be long, so that the column of water in it will exert suction and accelerate filtration. The end of the stem is ground to a bevel. After fitting the filter paper into the funnel the latter is filled with water and the paper is pressed against the funnel with the fingers so that an air-tight connection is obtained, and the column of water in the stem is not broken by leakage of air between the funnel and the paper. During filtration and washing the point of the beveled end of the stem should touch the side wall of the receiving beaker, but should not dip below the surface of the liquid in the receiver.

b. GLASS FUNNELS FOR FILTRATION WITHOUT WASHING. In analyses in which an aliquot of the filtrate is used the precipitate is thrown into a dry filter, and is not washed. The funnels used for

such filtrations are short stemmed, and do not need to meet the requirements of accurate 60° angles and straight sides. The most rapid filtrations are obtained with pleated filter papers.

c. PORCELAIN BUCHNER FUNNELS.

- (1) The Buchner funnel has vertical walls, and a perforated flat surface to support a filter paper. The chief use of the Buchner filter is for filtration of precipitated reagents; it is not used when the precipitate is to be weighed, nor, as a rule, when an aliquot of the filtrate is to be analyzed.
- (2) For use, the filter plate is fitted with a hardened filter paper, or with two or three ordinary filter papers to withstand the suction, and the paper is wet with a little water or supernatant solution from the mixture to be filtered. The stem of the funnel, fitted into a perforated rubber stopper or section of rubber tubing, is fitted into the neck of a suction flask, and the wet filter paper is drawn tight to the perforated bottom by starting the suction. A properly fitted paper lies completely flat on the perforated plate, covering all the perforations and extending nearly to the side walls. The stopper must be large enough to prevent its being drawn through the neck of the flask by great pressure. The mixture to be filtered is poured into the funnel and the filtration is continued with suction. With precipitates that tend to clog the pores of the filter paper it is advisable to apply suction gently at first. As soon as all the supernatant is drawn through the funnel the latter is filled with washing liquid, which is completely drawn through before another portion is poured into the funnel. For efficient washing it is necessary to avoid the formation of cracks in the precipitate, because the washing fluid will be drawn through the cracks instead of perfusing the precipitate. Therefore, after wash liquid has been drawn off, do not continue the suction until the layer of precipitate shrinks and cracks. If a crack is seen to form, it is pressed together with a footed rod and at once covered with washing fluid.
- (3) After the washing is finished the precipitate can be partially dried by drawing air through it. If washing with aqueous solution is followed by washing with alcohol and ether, air-drying may yield a completely dried product.

Section V. ACID-BASE INDICATORS

120. General

a. **PROTON DONORS AND ACCEPTORS.** The hydrogen atom is conceived to have two parts; the *proton*, symbol H^+ , which is the nucleus of the hydrogen atom, and which carries unit electrical charge of the kind designated by the positive sign, and the *electron*, which is characterized as the unit electrical charge of the kind designated by the negative sign. Any one of the common acids is a proton donor. The transfer of a proton from the carboxyl group of acetic acid, $CH_3.COOH$, to another substance leaves the anion of acetic acid, $CH_3.COO^-$, which may be called a proton acceptor. The primary anion $H_2PO_4^-$, of phosphoric acid is both a proton acceptor, capable of forming H_3PO_4 and a proton donor, capable of forming HPO_4^- . The ammonium ion, NH_4^+ , and substituted ammoniums are proton donors. Instead of using various names for the several classes of material it is convenient to unify the treatment by use of the terms *proton donor* and *proton*

Table VIII. Conjugate Pairs of Proton Donors and Acceptors

Proton donors	Proton acceptors	pK'
H_3^+O or $H^+.H_2O$ (hydrogen ion of aqueous solution).	H_2O (water, simplest molecule)	*
H_2O (water, simplest molecule)	OH^- (hydroxyl ion)	*
$CH_3.COOH$ (acetic acid)	$CH_3.COO^-$ (acetate anion)	4.7
$H_2PO_4^-$ (primary phosphate anion)	HPO_4^- (secondary phosphate anion)	6.8
NH_4^+ (ammonium cation)	NH_3 (ammonia)	9.4
$CH_2NH_3^+$ (glycine cation) $COOH$	$CH_2NH_3^+$ (glycine dipole ion) COO^-	2.4
$CH_2NH_3^+$ (glycine dipole ion) COO^-	CH_2NH_2 (glycine anion) COO^-	9.8
H_3BO_3 (boric acid)	$H_2BO_3^-$ (primary borate anion)	9.1
In^- (yellow univalent anion of the indicator phenol red)	$In=$ (red bivalent anion of the indicator phenol red)	7.8

1
* The sum $pH + \log \frac{1}{[OH^-]}$ approximates 14.0.

acceptor. Examples of conjugate pairs of proton donor and acceptor are shown in table VIII.

b. NATURE OF THE HYDROGEN ION. It was assumed at one time that an acid typified by HA, dissociates in solution to a great or small degree in the sense: $HA \rightleftharpoons H^+ + A^-$. Thus it would appear that the hydrogen ion, H^+ , is a free proton. This is improbable. It is preferable to regard the ion as interacting with molecules of the solvent. If water is the solvent, the reversible process is represented by $HA + H_2O \rightleftharpoons A^- + H_3O^+$. The hydrogen ion, H_3O^+ , of a water solution thus appears as a hydrated proton. In any other solvent the hydrogen ion would be different. This emphasizes the necessity of avoiding the common mistake of making *direct* comparisons between measurements of pH (see below) in solutions of which the solvents are different. The following treatment is restricted to water solutions.

c. HYDROGEN ION CONCENTRATIONS AND ACTIVITIES.

- (1) Hydrogen ion concentrations are expressed in terms of normality. To simplify elementary theory with which to draw the bold outlines of the subject it is legitimate to make certain assumptions, which cannot be completely realized, and to use hydrogen ion concentrations in dealing with equilibria among proton donors and acceptors. Thus the product of the hydrogen ion concentration and the hydroxyl ion concentration of a water solution may be regarded as 1×10^{-14} and from this relation the concentration of the one may be calculated if the concentration of the other be known. A solution in which the hydrogen ion concentration equals the hydroxyl ion concentration is thus calculated to be $1 \times 10^{-7} N$ with respect to each of these ions. This concentration is frequently made an arbitrary point of demarkation between what are called "acid" solutions and "alkaline" solutions. There is no other practical use of this demarkation.
- (2) Actually the hydrogen ions in any solution of electrolytes are under the constraint of interionic forces which make them less available as proton donors than would appear from the value for the normal concentration in any given instance. Accordingly, the concentration is multiplied by a factor, called the *activity coefficient*, and the product is called the *activity*. For example, there is evidence that in a solution 0.1 molar with respect to hydrochloric acid the hydrogen ion concentration is 0.1 *N*. The activity coefficient is about 0.8 and pH (*d* below) is approximately 1.1.

d. PRACTICAL MEANING OF PH.

- (1) Originally pH was defined as the logarithm of the reciprocal of the hydrogen ion concentration. Thus, if the concentration were 1.0 N, $\text{pH} = 0.0$; if the concentration were 0.001 N, $\text{pH} = 3.0$, etc. Likewise, the arbitrary point of division between "acid" and "alkaline" solutions would be $\text{pH} = 7.0$. Actually the hydrogen cell, with the aid of which the basic data of the subject have been determined, provides no means of determining hydrogen ion concentrations. Were certain requirements to be met rigidly, the device would permit the determination of hydrogen ion activities. These, not the concentrations, determine the states of equilibria and therefore are the more important. A very close approximation may be made so that it might appear legitimate to redefine pH as the logarithm of the reciprocal of the hydrogen ion activity. Unfortunately the requirements cannot be met rigidly, so that an arbitrary element enters, due to the necessity of neglecting small and incalculable variables.
- (2) The standard buffer solutions given in paragraph 121 have had assigned to them pH values obtained by measurements with the hydrogen cell. The ultimate standards of reference are undergoing continual slight revision. In order to keep the data obtained with the use of these secondary standards in conformity with the data reported in the past by the Army Medical Service Graduate School no revision has been made.

e. A USEFUL EQUATION.

$$\text{pH} = \text{pK}' + \log \frac{\text{concentration of proton acceptor}}{\text{concentration of proton donor}} \quad (1)$$

In this equation log signifies logarithm to the base 10. pK' is characteristic of the particular pair of proton donor and acceptor, and may be considered to be a constant for a limited range of conditions, temperature, salt concentration of the solutions, etc. A true constant and the ratio of activity coefficients for proton acceptor and donor are included in pK' . The reason that a value of pK' can be used only for a limited range of conditions is that the ratio of activity coefficients changes with change of conditions. (See tables VIII and XI for pK' values that are valid for ordinary conditions.) When the concentrations of proton donor and acceptor are equal, $\text{pH} = \text{pK}'$. As the ratio of the concentration of acceptor to that of the donor increases, pH increases. When the ratio decreases, pH decreases. If this ratio is 9:1, pH is 0.95 unit

greater than pK' . If the ratio is 99:1, pH is about 2.0 units greater than pK' . If the ratio is 1:99, pH is about 2.0 units less than pK' .

f. NATURE OF BUFFERS. A buffer may be described crudely as a system that tends to stabilize the pH value of a solution against large changes following addition of acid or alkali. A simple example may be treated with the aid of equation (1), which happens to serve in the case selected.

Suppose a solution contains 0.05 M $HPO_4^{=}$, and 0.05 M $H_2PO_4^-$. According to equation (1) and the value of pK' given in table

VIII, $pH = 6.8 + \log \frac{0.05}{0.05}$. Whence, $pH = 6.8$. Now add to 1 liter

of this solution 10 ml of 0.1 M hydrochloric acid. This will convert some of the secondary phosphate to primary phosphate, and the

relation will be, $pH = 6.8 + \log \frac{0.05 - 0.001}{0.05 + 0.001}$. Whence, $pH = 6.78$.

Had the above amount of hydrochloric acid been added to 1 liter of pure water, the pH value would have been only slightly greater than 3.0.

As may be noted by inspection of equation (1), the stabilizing, or so-called "buffer effect" of such a system is greater when the ratio of the concentration of proton acceptor to that of the donor is unity. As the ratio departs in either direction from unity, the stabilizing effect becomes less, and ceases to have practical value when the ratio becomes very small or very large. Thus any one buffer system of proton donor and acceptor can be used practically only within a limited range of pH that may be defined arbitrarily as 1.5 (or less) units of pH greater and less than the value of pK' .

In addition to simple cases, such as the above, for which equation (1) is applicable, the water acts as a buffer at high and low pH. Proteins and other complex substances serve as buffers. To establish standards for the colorimetric determination of pH, to stabilize the pH values of physiologic solutions and culture media and for various other purposes, buffer solutions are invaluable.

g. INDICATORS. An "acid-base indicator" is a substance capable of forming a system of proton donor and acceptor at least one of which must have a distinctive color in solution. For example, the monosodium salt of phenol red (phenolsulonephthalein) provides the ion and proton donor which may be represented by HIn^- . A solution containing these ions appears yellow. Loss of proton yields the ion In^- . A solution containing these ions appears red. A solution containing a mixture of these two ions has a color that depends on the absorption of light by both forms.

Some indicators exhibit two color transformations, each occurring within a distinct range of pH. (See, for example, thymol blue, listed in table XI.)

If a means is provided for determining the *ratio* of the concentrations of the two forms of the indicator in a given solution, it is possible to estimate the pH value of the solution. A method in which this is done by comparison will be given.

As may be shown by inspection of equation (1) or by trial calculations, an indicator has a limited range of pH within which there appear appreciable differences in the color of its solution. Therefore, in the use of an indicator for the determination of pH, the first step is to select that indicator which is suitable for the solution under test. Practically it suffices to make preliminary trials with small portions of the tested solution and to use first an indicator of low pK' , then one of high pK' , and to narrow the selection systematically until there is found an indicator that exhibits a degree of transformation sufficient for the differentiation of small variations of pH, that is partly in each of its two forms at the pH of the solution to be tested. Useful ranges are given in table XI. Then proceed according to paragraph 122.

121. Preparation of Buffer Solutions

a. PREPARATION OF STOCK SOLUTIONS

- (1) *M/5 potassium chloride solution.* The salt should be recrystallized three or four times, and dried at 120° C. to constant weight. Use 14.912 gm* per liter of solution.
- (2) *M/5 acid potassium phthalate solution.* This salt should be crystallized from solutions at temperatures not lower than 20° C., and the *M/5* solution diluted to 0.05 *M* should have a pH value close to 4.0. Dry the crystals at 110° C. to constant weight. For the stock solution use 40.836 gm per liter of solution.
- (3) *M/5 acid potassium phosphate solution.* A 0.025 *M* solution of this salt should show no turbidity on long standing. The salt should be dried at 110° C. to constant weight. The stock solution should contain 27.232 gm per liter of solution. The solution should be distinctly red with methyl red and distinctly blue with brom phenol blue.
- (4) *M/5 boric acid, M/5 potassium chloride solution.* Boric acid should be recrystallized several times from distilled

*The quantities of materials specified for making up these *M/5* solutions were calculated in accordance with the atomic weights assigned at the time the measurements of pH were made. The specified quantities should be used and not modified in accordance with subsequent changes in atomic weights.

water, and dried to constant weight at room temperature in a desiccator over calcium chloride. One liter of the stock solution should contain 12.405 gm* of boric acid and 14.912 gm of potassium chloride.

(5) *M/5 sodium hydroxide solution.* For the preparation of sodium hydroxide solutions free from carbonate and their standardization, see paragraph 136.

(6) *M/5 hydrochloric acid solution.* For preparation and standardization, see paragraph 133.

b. COMPOSITION OF BUFFER SOLUTIONS. The compositions of various buffer solutions are indicated in table IX. It should be noted that the pH scale is constantly undergoing slight revision.

122. Colorimetric Determination of pH

a. COMPARISON WITH BUFFER STANDARDS. This is the preferred method.

(1) *General.* The fundamental assumption is that standard and tested solutions have the same pH when each produces the same color with the same indicator. Accordingly, the procedure is one of color comparison between the solution to be tested and the standard, and for this the following specifications are essential:

(a) The tested solution must have a buffer action sufficient to prevent the added indicator from making a significant change in pH. (This seldom has to be considered except for solutions of neutral salts, distilled water, or greatly diluted biologic solutions, for which special methods must be used.)

(b) The indicator must be so chosen that it shows significant color changes with small changes in pH.

(c) The standard solution and the solution to be tested must contain the same indicator at the same concentration.

(d) Each solution must be viewed through the same length of absorbing column, and the incident light must be of the same intensity. (When standard solutions containing the indicator are provided, this specification and (c) above must be met with particular care so that the stock solution of the indicator added to the test solution will provide the indicator concentration specified for the standards and so that the tubes used have the internal diameter of the tubes containing the standards.)

*The quantiles of materials specified for making up these M/5 solutions were calculated in accordance with the atomic weights assigned at the time the measurements of pH were made. The specified quantities should be used and not modified in accordance with subsequent changes in atomic weights.

Table IX. Composition of Buffer Solutions Giving pH Values at 20°C†

A

0.2M KCl	0.2M HCl	Final volume	pH
ml	ml		
0.00	59.5	Dilute to 100 ml.....	1.0
2.72	47.28	Dilute to 100 ml.....	1.1
12.45	37.55	Dilute to 100 ml.....	1.2
20.16	29.84	Dilute to 100 ml.....	1.3
26.30	23.70	Dilute to 100 ml.....	1.4
31.18	18.82	Dilute to 100 ml.....	1.5
35.03	14.95	Dilute to 100 ml.....	1.6
38.12	11.88	Dilute to 100 ml.....	1.7
40.57	9.43	Dilute to 100 ml.....	1.8
42.51	7.49	Dilute to 100 ml.....	1.9
44.05	5.95	Dilute to 100 ml.....	2.0
45.27	4.73	Dilute to 100 ml.....	2.1
46.24	3.76	Dilute to 100 ml.....	2.2

B

0.2M KH phthalate	0.2M HCl	Final volume	pH
ml	ml		
50	46.60	Dilute to 200 ml.....	2.2
50	39.60	Dilute to 200 ml.....	2.4
50	33.00	Dilute to 200 ml.....	2.6
50	26.50	Dilute to 200 ml.....	2.8
50	20.40	Dilute to 200 ml.....	3.0
50	14.80	Dilute to 200 ml.....	3.2
50	9.95	Dilute to 200 ml.....	3.4
50	6.00	Dilute to 200 ml.....	3.6
50	2.65	Dilute to 200 ml.....	3.8

C

0.2M KH phthalate	0.2M NaOH	Final volume	pH
ml	ml		
50	0.40	Dilute to 200 ml.....	4.0
50	3.65	Dilute to 200 ml.....	4.2
50	7.35	Dilute to 200 ml.....	4.4
50	12.00	Dilute to 200 ml.....	4.6
50	17.50	Dilute to 200 ml.....	4.8
50	23.65	Dilute to 200 ml.....	5.0
50	29.75	Dilute to 200 ml.....	5.2
50	35.25	Dilute to 200 ml.....	5.4
50	39.70	Dilute to 200 ml.....	5.6
50	43.10	Dilute to 200 ml.....	5.8
50	45.40	Dilute to 200 ml.....	6.0
50	47.00	Dilute to 200 ml.....	6.2

Table IX. Composition of Buffer Solution Giving pH Values at 20°C†
— Continued

D

0.2M KH ₂ PO ₄	0.2M NaOH	Final volume	pH
ml	ml		
50	3.66	Dilute to 200 ml.....	5.8
50	5.64	Dilute to 200 ml.....	6.0
50	8.55	Dilute to 200 ml.....	6.2
50	12.60	Dilute to 200 ml.....	6.4
50	17.74	Dilute to 200 ml.....	6.6
50	23.60	Dilute to 200 ml.....	6.8
50	29.54	Dilute to 200 ml.....	7.0
50	34.90	Dilute to 200 ml.....	7.2
50	39.34	Dilute to 200 ml.....	7.4
50	42.74	Dilute to 200 ml.....	7.6
50	45.17	Dilute to 200 ml.....	7.8
50	46.85	Dilute to 200 ml.....	8.0

E

0.2M H ₃ BO ₃ 0.2M KCl	0.2M NaOH	Final volume	pH
ml	ml		
50	2.65	Dilute to 200 ml.....	7.8
50	4.00	Dilute to 200 ml.....	8.0
50	5.90	Dilute to 200 ml.....	8.2
50	8.55	Dilute to 200 ml.....	8.4
50	12.00	Dilute to 200 ml.....	8.6
50	16.40	Dilute to 200 ml.....	8.8
50	21.40	Dilute to 200 ml.....	9.0
50	26.70	Dilute to 200 ml.....	9.2
50	32.00	Dilute to 200 ml.....	9.4
50	36.85	Dilute to 200 ml.....	9.6
50	40.80	Dilute to 200 ml.....	9.8
50	43.90	Dilute to 200 ml.....	10.0

(e) If the solution to be tested is colored or turbid, the effect thereof must be compensated. Compensation may be obtained by means of the block comparator.

(2) *Method.* Place the tube containing test solution and indicator in one hole of the comparator and back it with a water blank. At one side place that standard plus indicator which is judged nearly to match the test solution, and back it with a tube containing a portion of the un-

†It is important to check the consistency of any particular set of these mixtures by comparing 5.8 and 6.2 phthalate with 5.8 and 6.2 phosphate, using brom cresol purple. Also 7.8 and 8.0 phosphate should be compared with the corresponding borates, using cresol red.

treated solution under test. The absorption of light is then that of the indicator plus that of the natural color of the test solution. Beside the test solution place a similar pair of tubes, one of which is another buffer standard of pH lower or higher than the first. Change standards until a color-match is obtained or until it is judged that an interpolated pH number can be assigned to the test solution. This device serves fairly well for solutions of moderate turbidity.

- (3) *Sources of error.* The primary assumption that two solutions have the same pH when their colors match under the conditions specified in (2) above, is not always adequate. The standard and the test solution may have the same color and yet differ in pH number if one solution differs much from the other in total salt concentration. Also the pH numbers assigned to buffer standards are strictly valid only for a specified temperature. The values of pK' given in table XI are strictly valid only for a temperature of 20° C. and for solutions the total salt concentrations of which are near those of the standard buffer solutions. A value of pK' is the sum of a true constant and variable quantity dependent in a complicated way on temperature and on all the ions present. Indicators may also combine with proteins or they may be carried out of the field of action by the formation of complexes with metal ions, by absorption on particulate matter, etc.
- (4) *Accuracy.* For many empirical uses in which no attempt is made to analyze the chemical detail and in which the emphasis is on a correlation between pH and an end-result, it is of less importance to attempt the often uncertain corrections for accuracy than to report precise measurements made according to the standard procedure here described. If the case demands an accuracy requiring corrections of the apparent readings, advanced texts should be consulted.

b. **DETERMINATION WITHOUT STANDARDS.** The following method requires no buffer solutions. It may be used in an emergency when standard buffers are not available. Provide test tubes of equal bore by selecting those in which the columns of water are very nearly of equal height when each contains exactly 10 ml of water. The variation in height of column should be less than 3 percent. Mark each tube at the meniscus before discarding the water. Consider the specific case in which a preliminary trial shows that phenol red is the proper indicator to use. Add 10 drops of a 0.04 percent so-

lution of phenol red to one of the tubes, and add the solution under test to the 10-ml mark. Mix well. *Assume*, for example, that phenol red has been transformed so that the ratio of the concentration of the red form to that of the yellow form is 2:8. Whence by the specific application of equation (1), paragraph 120*e*,

$$\text{pH} = 7.8 + \log \frac{(\text{red form})}{(\text{yellow form})} = 7.8 - 0.6 = 7.2 \quad (2)$$

To ascertain that this degree of color transformation has taken place it is necessary to obtain a color-match with known ratios of the two forms. This is accomplished as follows. To one tube add 2 drops of the 0.04 percent phenol red solution and dilute to the 10-ml mark with water made sufficiently alkaline (0.01 *N* NaOH) to convert *all* the indicator to the red form. To another tube add 8 drops of the 0.04 percent phenol red solution and dilute to the 10-ml mark with water made sufficiently acid (0.001 *N* HCl) to convert all the indicator to the yellow form. Avoid excess acid.

Caution: All indicators of the general structure of phenol red (sulfonephthaleins) exhibit a second color transformation at very low pH. (See table XI for the case of thymol blue, which is used to determine low pH values as well as high ones.)

When these two reference tubes are viewed in tandem the resultant color should match that of the test solution. Although the 10 drops of indicator solution in the two comparison tubes are distributed in 20 ml, whereas the 10 drops of indicator solution are in only 10 ml of the test solution, the length viewed in the first case is twice that of the second. (See Beer's law.)

Instead of apportioning the indicator by measured drops, it may be apportioned by volumes.

It is well to use the comparator block and to back the unknown by a water blank. If the tested solution is colored to begin with, the principle previously described may be applied with a comparator block accommodating sets of three tubes in tandem — the tube with acidified indicator, the tube with alkalinized indicator, and a tube with test solution without indicator in one row and the tube with test solution plus indicator backed by two water blanks in another row.

- (1) Of course it cannot be predicted that the test solution will convert the indicator to the 2:8 ratio as *assumed* in the above example. Accordingly, there is prepared a judicious selection of several pairs of comparison tubes with the apportionments of 10 drops of phenol red solution shown in table X. These pairs, the tubes of which are viewed in tandem in the comparator block, are inter-

changed until a color-match with the tested solution is found or approximated.

- (2) Table X and the following equation will serve for any of the sulfonephthaleins listed in table XI. The sulfonephthaleins may be identified in table XI as those for which a number is given in the last column. For the calculation of the pH value use the value of pK' (table XI) for the indicator chosen, the log ratio as found in table X, and the following equation:

$$pH = pK' + \log \frac{\text{drops of indicator solution per 10 ml of alkalinized solution}}{\text{drops of indicator solution per 10 ml of acidified solution}} \quad (3)$$

If it is necessary to use other apportionments of indicator in comparison tubes, the log ratios may be found by use of a table of logarithms. It also may be advantageous to use a more dilute solution of indicator. For precise measurement the indicator solution is apportioned by volume.

The paired tubes of indicators may be labeled, kept, and used as pH standards. If protected from light, in pyrex glass, some of them, notably phenol red, will remain unchanged for months. Others, notably the halogenated compounds, may fade in alkaline solution. Commercial comparators using colored discs are available.

123. Use of Indicators in Titrations

a. When a solution of an acid is titrated with a standard solution of an alkali, it is of crucial importance to determine equality

Table X. Composition of pH Standard Solutions

Pair	a	b	c	d	e	f	g	h	i
Number of drops of indicator solution per 10 ml of alkalinized solution.....	1	2	3	4	5	6	7	8	9
Number of drops of indicator solution per 10 ml of acidified solution.....	9	8	7	6	5	4	3	2	1
Log ratio to be added to pK'	-0.95	-0.60	-0.37	-0.18	0	0.18	0.37	0.60	0.95

in the stoichiometrical equivalents of acid and base (alkali). The so-called "equivalence point," or "end-point," is characterized by a pH number that can be detected by means of an indicator. Ideally the pH of the equivalence point is unique for each case. It depends principally on the so-called "strengths" (as measured by pK' for weak acids) of the titrated system and reagent and on the dilution.

b. In the titration of a strong acid (HCl or H_2SO_4) by a strong alkali ($NaOH$ or KOH) the ideal end-point is pH 7.0, which may be detected with phenol red, but except for very dilute solutions and for extreme precision, a latitude of 1.5 units pH will make an insignificant error. If the precision is to be extremely high in any case, allowance must be made for the slight buffer effect of the indicator. For weaker acids the pH of the equivalence-point is the higher the higher the pK' . The equivalence-point cannot be calculated with equation (1) alone because a very weak proton donor does not yield that proportion of its available protons which corresponds to the proportion of added alkali. For example, in the case of boric acid, the stoichiometrical equivalence-point is reached before all the boric acid is converted to borate ions.

c. For all acids with pK' values less than 6 a fair approximation, sufficient for ordinary purposes, is to titrate to pH 8.5, which purpose phenolphthalein serves well. Advantage is also taken of the fact that the last small addition of alkali (split drop) makes a large change of pH. Therefore, when phenolphthalein is the indicator, the solution remains colorless, except at local regions where mixing is incomplete, until the end-point is closely approached, and just at the last, the small addition of alkali necessary to attain the end-point changes the pH so much that it transforms a readily detectable portion of the indicator to red. In all titrations with phenolphthalein it is necessary to guard against the effect of atmospheric CO_2 . Also, the alkali is always added from a burette to the acid in a flask, so that the solution in the flask will be acid up to the last moment of the titration. A standard solution of alkali should always be protected against entrance of CO_2 .

d. For acids of pK' higher than 6 it is necessary to be the more precise the higher the pK' , and, as in the case of boric acid, it may be necessary to calculate the required pH accurately and to ascertain when it is attained by comparison with a standard.

e. When ammonia is absorbed in a standard solution of hydrochloric or sulfuric acid, and the residual acid is titrated to determine the amount of ammonia absorbed, consideration must be given to the following: The absorbed ammonia has become ionized (NH_4^+). If the pH is raised too high, protons will be stripped from these ions, and ammonia will be regenerated, requiring standard alkali in addition to that necessary to titrate the residual

acid. Accordingly, the end-point is not pH 7.0, as in titrating the acid alone, but must be considerably lower. If the ammonium ion is at 0.1 *N*, the end-point is about pH 5.0, detectable by bromcresol green or methyl red. For more dilute solutions the end-point is appreciably higher. It is about pH 5.5 for 0.01 *N* ammonium ions, and pH 6.0 for 0.001 *N*, the latter being a fairly rigid requirement allowing little latitude. In these cases, chlorphenol red is preferred, although bromcresol green may be used.

f. If the titration of ammonia held by an excess of boric acid, as in one method, the pH value of the original boric acid solution should be restored, and for this purpose it is best to set up a standard.

g. There are several cases in which titration to an arbitrarily selected indicator-change has only the significance that the titer is a measure of total buffer effect. The titration of gastric juice to pink with phenolphthalein is a case in point. The empirical uses of the data in such cases depend on judgment of how far the specific case departs from a norm.

h. Reliable indicators are listed in table XI. Litmus (azolitmin) is an indicator of variable composition and is not reliable for precise measurements. Its pH range is roughly: red, 4.5 — 8.3, blue. Litmus paper is convenient for noting whether a solution is distinctly acid or alkaline.

Section VI. OXIDATION-REDUCTION REACTIONS

124. Nature of Oxidation-reduction Reactions

Fundamentally, an oxidation-reduction reaction consists of a shift of electrons, or negative charges, from a reducing agent to an oxidizing agent. These reactions, as employed in quantitative analysis, may be divided into two types.

a. The first type is empirical. Analytical methods of this class are based on oxidation-reduction reactions that have not been defined in stoichiometric terms. Examples of such methods comprise nearly all the titrimetric and colorimetric procedures for the determination of reducing sugars (par. 202), and the procedure of Fiske and Subbarow for the determination of inorganic phosphate (par. 210).

b. The second type includes procedures that are defined stoichiometrically. An oxidation-reduction reaction to be placed in this class must satisfy certain criteria that have been imposed by theory and experimentation. Under proper conditions, such reactions must proceed practically to completion according to a well-defined process when equivalent amounts of the reacting sub-

stances are present. If the reaction is used in a titration, it must have also the following properties: it must be practically instantaneous, and the end-point (equivalence-point) must be defined sharply by some change in the physical or chemical properties of the solution, such as change in color. If the reagents themselves do not provide a means of detecting the end-point, it must be possible to add a third substance, an indicator, which reacts only when the main reaction is complete. This type of precise volumetric analysis requires consideration of various details, which are presented in the following discussion.

Table XI. Characteristics of Indicators

Indicator	pK'	pH-range and colors	ml 0.01 N NaOH per 0.1 gm ^a
Thymol blue (acid range) -----	1.7	Red, 1.2—2.8, yellow--	21.5
Methyl yellow ^b -----	3.3	Red, 2.9—4.0, yellow--	(c)
Methyl orange ^d -----	3.5	Red, 3.1—4.4, yellow--	(e)
Bromphenol blue -----	4.0	Yellow, 3.1—4.7, blue--	14.9
Bromcresol green -----	4.7	Yellow, 3.8—5.4, blue--	14.3
Methyl red -----	5.0	Red, 4.2—6.3, yellow--	(f)
Chlorphenol red -----	6.0	Yellow, 5.1—6.7, red--	23.6
Bromcresol purple ^e -----	6.2	Yellow, 5.4—7.0, purple	18.5
Bromthymol blue -----	7.1	Yellow, 6.1—7.7, blue--	16.0
Phenol red -----	7.8	Yellow, 7.0—8.6, red--	28.2
Cresol red -----	8.3	Yellow, 7.4—9.0, red--	26.2
Thymol blue (alkaline range) ----	8.9	Yellow, 8.0—9.6, blue--	21.5
Phenolphthalein ^h -----	9.7	Colorless, 8.3—10.0, red	(i)

^a To make a 0.04 percent stock solution of an indicator for which a number is given in the last column grind 0.1 gm of the pure, acid indicator with the number of milliliters of 0.01 N NaOH directed and when solution is complete, dilute to 250 ml with distilled water.

^b Methyl yellow (Töpfer's indicator) (Pdihethylaminoazobenzene) is used in measurements of gastric acidity.

^c Stock solution of methyl yellow: 0.01 gm + 0.1 ml 0.1 N HCl + 80 ml 95 percent ethyl alcohol + 20 ml water.

^d Do not use with phthalate buffers.

^e Stock solution of methyl orange: 0.05 gm in 100 ml water.

^f Stock solution of methyl red: 0.02 gm in 60 ml 95 percent ethyl alcohol; add 40 ml water.

^g Beware of dichromatic effect when used to determine pH of turbid solution.

^h Fades in strong alkali.

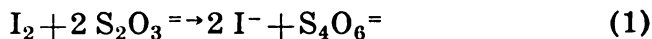
ⁱ Stock solution of phenolphthalein: 1 gm in 100 ml 95 percent ethyl alcohol; more dilute solutions are made by diluting the stock solution.

125. Calculation of Equivalent Weight of an Oxidizing or Reducing Agent

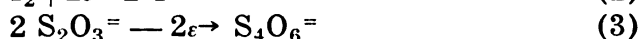
a. It is convenient to employ a unit of concentration for solutions of oxidizing or reducing agents that provides a simple relation to solutions of other oxidizing and reducing agents, regardless of their nature. This is attained by expressing concentrations in terms of a *normal* solution, or multiples or fractions thereof. A

normal solution is one that contains 1 gram-equivalent weight of solute per liter of solution. The definition for a gram-equivalent weight of an oxidizing or reducing agent is based on the fundamental fact that an oxidation-reduction reaction consists of an exchange of electrons. The gram-equivalent weight of an oxidizing or reducing agent is equal to the gram-molecular weight of the compound divided by the number of electrons exchanged per molecule *in the particular oxidation-reduction reaction involved*. Information concerning a particular reaction employed in volumetric analysis must be based on previous experimentation and can be obtained by reference to standard textbooks of quantitative analysis. (See app.)

b. To illustrate the utilization of such information, one may consider the titration of a solution of iodine (I_2) by means of a solution of sodium thiosulfate ($Na_2S_2O_3$). The essential features of the reaction involved can be represented according to the following ionic equation:



c. The accompanying cations, Na, remain unchanged in the reaction, hence they are omitted from equation (1). Evidently in reaction (1) negative electric charges have changed position. The unit charge is the electron. For the purpose of examining the electronic exchange involved in reaction (1), it may be separated into two half-reactions:



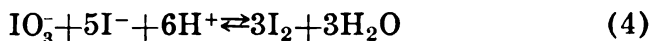
where e represents the electron. These equations provide a balance-sheet by which one may account for the various atoms, ions, and electric charges. Of great importance is the fact that they permit the calculation of the equivalent weights of the reactants. From equation (2) it may be seen that the gram-equivalent weight of iodine for this reaction is equal to the gram-atomic weight, inasmuch as one electron is accepted by each iodine atom. Equation (3) indicates that one electron is donated by each thiosulfate ion, therefore the equivalent weight of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), for this reaction, is equal to the molecular weight. Other examples of oxidation-reduction reactions are given below, and additional reactions and half-reactions are specified in textbooks.

d. It must be emphasized again that in order to describe an oxidation-reduction reaction in terms of chemical equations, such as those presented below, it is necessary to know the products of the reaction under the *particular conditions employed in the determination*. Such information has been acquired by careful and

thorough experimentation. Usually it is necessary to control precisely the conditions for a given oxidation-reduction reaction in order that it may be applied in quantitative analysis. Therefore, in examining the following equations, it should be recognized that carefully defined conditions are implied. The equations are presented merely as a convenience for the calculation of stoichiometric equivalents of the reactants for the *particular conditions described in the practical sections of this manual*.

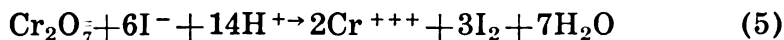
126. Examples of Oxidation-reduction Reactions and Their Application

a. STANDARDIZATION OF THIOSULFATE WITH IODATE. In acid solution iodates (IO_3^-) oxidize iodides according to the following reaction:



Thus, there is produced by the action of an iodate ion upon an excess of iodide (in the presence of acid) *six* atoms of iodine, hence the equivalent weight of an iodate (such as KIO_3) is one-sixth of the grammolecular weight. This reaction, in conjunction with the reaction of equation (1), is employed in the determination of chloride by the method of Sendroy. (See par. 209.)

b. STANDARDIZATION OF THIOSULFATE WITH POTASSIUM DICHROMATE (par. 141). For this standardization, iodide in an *acid solution* is oxidized to iodine by means of primary standard dichromate ($\text{Cr}_2\text{O}_7^{2-}$) according to the following equation:

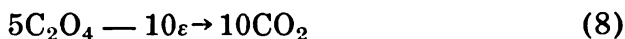
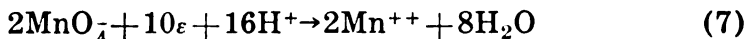


The iodine produced in reaction (5) then is titrated with thiosulfate according to reaction (1). Inasmuch as *six* iodine atoms are produced by the action of *one* dichromate ion upon an excess of iodide (in the presence of acid) as represented in equation (5), the equivalent weight of potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) *for this reaction* would be equal to one-sixth of the molecular weight.

c. STANDARDIZATION OF OXALIC ACID WITH PERMANGANATE. In the titration of oxalic acid with permanganate the following reaction occurs:



This reaction can be represented according to two half-reactions:



From equation (7), the equivalent weight of potassium permanganate (KMnO_4) is one-fifth the molecular weight inasmuch as

each permanganate ion accepts five electrons. The equivalent weight of sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$) is one-half the molecular weight (from equation 8). This reaction is employed in the determination of calcium in serum by the method of Clark and Collip.

Section VII. VOLUMETRIC TITRATIONS AND SOLUTIONS

127. Reactions and Technics of Volumetric Analysis

a. REACTIONS. In a volumetric titration the amount of Substance A, which is to be determined, is estimated from the volume of a standard solution of Substance B required to react quantitatively with A. The standard solution of Substance B is added from a burette until the end-point (par. 130) is reached. Various reactions are used in volumetric titrations, but the methods described in this manual employ either *acid-alkali* reactions (par. 123) or *oxidation-reduction* reactions (pars. 124 and 125.)

b. TECHNICS. The technics of handling volumetric flasks, burettes, pipettes, and cylinders are discussed in section III of this chapter. Gross errors can occur unless proper technic is employed.

128. Definition of Molar and Normal Solutions and of Normality Factors

a. MOLAR SOLUTION. (Abbreviation: *M*). A molar solution of a substance contains 1 gram-molecule, or *mole*, of the substance per liter. The molecule weight of NaCl being 58.454, a molar solution contains 58.454 grams of NaCl per liter.

b. NORMAL SOLUTION (Abbreviation: *N*). A normal solution is one which contains per liter 1 gram-equivalent of the reacting solute. In an *acid-alkali* titration a gram-equivalent is the weight in grams of a substance which will, if an acid, donate, or if an alkali, accept, the protons of 1.008 gm of hydrogen under the conditions specified. (See par. 123.) For HCl and NaOH a gram equivalent is 1 mole. For H_2SO_4 and $\text{Ba}(\text{OH})_2$ a gram-equivalent is $\frac{1}{2}$ mole. For H_3PO_4 titrated with NaOH to NaH_2PO_4 (pH 4.4) a gram-equivalent is 1 mole, but when titrated to Na_2HPO_4 , with the end-point at pH 9, a gram-equivalent of H_3PO_4 is $\frac{1}{2}$ mole. In *oxidation-reduction* titrations a gram-equivalent of any reagent is 1 mole divided by the total number of electrons exchanged per molecule of the reagent in the particular reaction involved in the titration. (See par. 125.)

c. NORMALITY FACTOR OF STANDARD SOLUTION. The meaning of the "normality factor" is indicated by an example. If a solution designated as 0.1 *N* is found on standardization to be 0.1009 *N*.

it is 1.009 times its designated concentration and its "tenth-normal factor" is 1.009. The factor 1.009 is the number by which any volume of this solution must be multiplied to calculate the equivalent volume of exactly 0.1 *N* solution. In general, if *F* is the tenth-normal factor of a solution, *V* ml of the solution represent $F \times V$ ml of exactly 0.1 *N* solution; and if *F* is the hundredth-normal factor of a solution, *V* ml represent $F \times V$ ml of exactly 0.01 *N* solution, etc.

129. Primary and Secondary Standards and Their Use in Preparing Standard Solutions

a. DEFINITIONS.

- (1) A *primary standard* is an accurately titratable material, of exact composition, which can be precisely weighed under laboratory conditions. A desirable property is a large equivalent weight; this contributes to accuracy in weighing.
- (2) A *secondary standard* is a substance suitable for standard solutions, but not for precise weighing; hence its solutions must be standardized by titration against a primary standard. The majority of standard solutions employed in routine analyses are prepared from secondary standards, because the qualities of convenience and economy required in routine analysis are not often combined with the properties required of a perfect primary standard.

b. PREPARATION OF STANDARD SOLUTIONS OF PRIMARY STANDARDS. The theoretical weight of primary standard is accurately weighed, quantitatively transferred to a volumetric flask, dissolved, and the solution is diluted to volume, and thoroughly mixed.

c. PREPARATION AND STANDARDIZATION OF STANDARD SOLUTIONS OF SECONDARY STANDARDS.

- (1) *Preparation.* A preliminary solution is usually first prepared, somewhat stronger than the desired normality, and its normality factor is determined by titration against a primary standard. The preliminary solution is then diluted to the exact extent calculated to attain the desired normality, as described in paragraph 132. The accuracy of the final solution is checked by again titrating against the primary standard. The titrations against the primary standard may be done by either of the following two technics:

(2) *Standardization.*

- (a) *Titration against weighed portions of primary standard.* Portions of the primary standard substance, sufficient to react with 20 to 25 or 40 to 50 ml of the solution are accurately weighed and dissolved, then titrated with the solution of the secondary standard, delivered from a 25- or 50-ml burette. The normality factor, F , of the secondary standard solution is calculated as

$$F = \frac{\text{mg of } P_r}{(\text{equivalent weight of } P_r) \times N_d \times (\text{ml of } S)}$$

P_r is the primary standard, S is the solution of secondary standard, N_d is the designated normality (for example, 0.1) of the secondary standard.

In this procedure the only error in measuring the primary standard is the error of the weighing, which can be kept to less than 1 part per 1,000 if more than 100 mg of primary standard is weighed, as is usually the case when the normality of the solution of secondary standard is 0.1 or more.

- (b) *Titration against standard solutions of primary standard.* Portions of a primary standard solution, of the normality designated for the secondary standard, are measured from a transfer pipette calibrated to 1 per 1,000 accuracy, and are titrated with the solution of secondary standard; or the secondary standard solution may be measured from a pipette and titrated with the solution of the primary. The normality factor, F , of the secondary solution is calculated as:

$$F = \frac{\text{ml of solution of primary standard}}{\text{ml of solution of secondary standard}}$$

In this procedure the measurement of the primary standard is subject to the combined errors of the preparation of the primary standard solution (errors of weighing and diluting to volume) plus the error of measuring the portion of primary solution titrated. If each of these three errors should be 1 part per 1,000, the total possible error would be 3 parts per 1,000. Standardization against solutions of primary standards is generally less exact than standardization against weighed portions, unless the solution standardized is considerably more dilute than 0.1 N .

d. ACID AND ALKALI STANDARDS.

- (1) *Primary acid standards.* *Constant boiling hydrochloric acid solution* is cheap, easy to prepare, and has a composition which can be fixed with an accuracy within 1 part per 10,000. Its volatility is so low that it can be weighed to 0.1 mg in an open Erlenmeyer flask, even in the small amounts (for example, 300 to 400 mg) required for individual titrations to standardize 0.1 N alkali. It can be used with end-points at all pH's employed in acid-alkali titrations. Constant boiling HCl is the primary acid standard of choice when facilities for preparing it by distillation are available. The equivalent weight of the solution is given in table XII. *Potassium acid phthalate* ($\text{KHC}_8\text{H}_4\text{O}_4$, eq. wt. 204.2) (Bureau of Standards grade of purity) is also an excellent standard. It has the limitation that it requires an end-point at pH₈ or higher (phenolphthalein). Hence if significant amounts of CO_2 are present in the alkali solution titrated, inaccuracy results unless the CO_2 is removed by boiling as the end-point is approached. *Potassium bi-iodate* [$\text{KH}(\text{IO}_3)_2$, eq. wt. 389.94] has excellent properties; its high equivalent weight facilitates accurate weighing in standardization of dilute alkali solutions against weighed portions of the bi-iodate.
- (2) *Secondary acid standards.* *Sulfuric acid* (H_2SO_4 , eq. wt. 49.04), because of its cheapness and the stability of its solutions, is most practical for acid standard solutions used in routine analyses. (Hydrochloric acid is not quite so stable.)
- (3) *Primary alkali standards.* *Anhydrous sodium carbonate* (Na_2CO_3 , eq. wt., 53.00) is used as a primary alkali standard, but unless kept securely protected from the atmosphere it is likely to absorb both moisture and CO_2 ; either makes it inaccurate for use as a primary standard. Heating to 110° C. drives off the moisture, but 300° C. is required to change back to Na_2CO_3 any NaHCO_3 formed from exposure to the atmosphere. End-points at pH 6 or lower are used, and when an apparent end-point is approached by addition of acid, it is necessary to boil off the CO_2 before titrating to the true end-point. *Sodium tetraborate (borax)* ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, eq. wt. 190.7 with end-point at pH about 5.) is accurate if there is entire certainty that the water of crystallization accords with the formula, but unless an analyzed preparation is available the composition may be uncertain. In view of the

drawbacks in use of the commonly available primary alkali standards, it is usually more reliable to standardize a solution of a secondary acid standard, such as sulfuric acid, by comparison with a standard solution of HCl, as described in paragraph 134, or by similar comparison with acid phthalate or bi-iodate standard solutions.

e. OXIDIZING AND REDUCING STANDARDS.

- (1) *Primary oxidizing standards.* Potassium iodate (KIO_3 , eq. wt. 35.67), potassium bi-iodate $\text{KH}(\text{IO}_3)_2$, eq. wt. 32.50) and potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$, eq. wt. 49.04) have satisfactory properties. Potassium dichromate can be dried at 200°C . The iodates are convenient for routine analyses, as well as for standardizing other solutions.
- (2) *Secondary oxidizing standards.* Potassium permanganate (KMnO_4 , eq. wt. 31.60) and ceric sulfate ($\text{Ce}(\text{SO}_4)_2$, eq. wt. = mol. wt. 332.25).
- (3) *Primary reducing standards.* Arsenious oxide. (As_2O_3 , eq. wt. 49.46), sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$ eq. wt. 67.00), and pure electrolytic iron (eq. wt. 18.61), are used to standardize solutions of secondary oxidizing standards, such as permanganate and ceric sulfate, but are not much used in routine analyses.
- (4) *Secondary reducing standards.* The reducing standard used in the greatest variety of oxidation-reduction analyses is sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, eq. wt. = mol. wt. = 248.19). In routine biochemical analyses it is almost the only reducing standard employed. It is standardized against potassium iodate or dichromate.

130. End-points and Indicators

a. The end-point, or equivalence-point, of a titration is reached when exactly equivalent amounts of the two reagents have been mixed. The end-points in some titrations are detectible without addition of indicators; thus, in the titration of 0.1 *N* iodine solution by 0.1 *N* sodium thiosulfate, the end-point can be detected by the disappearance of the yellow color. In many titrations, however, it is necessary to add an indicator, which reacts with a slight excess of one of the reagents to produce a visible change. Thus, in the titration of highly dilute iodine solutions, such as 0.01 *N*, by sodium thiosulfate, the disappearance of the iodine color is not sharp, and starch indicator is added. Acid-base indicators have already been discussed. (See par. 120 through 124.)

b. In some, but not all, titrations, a slight excess of the reagent added from the burette is necessary to make the end-point visible. However, when the standard solution used is 0.1 *N* or stronger, the excess required to give the end-point, even in titrations where an excess is theoretically needed is usually negligible. When titrations requiring an excess are done with more dilute solutions, such as 0.01 *N*, it may be necessary to perform control titrations on water or suitable solutions to determine how much excess of standard solution is required to give the end-point; this amount is subtracted as a correction in each analysis.

c. The amount of each indicator to add is determined by trial. A minimum of indicator is likely to be desirable when the indicator changes from one color to another. When, on the other hand, the change is from colorless to colored a large excess of indicator may make the end-point visible with minimal excess of reagent. In acid-base titrations with the colorless-to-red change of phenolphthalein, when the desired pH at the end-point is on the lower edge of the red range at pH 8.3-8.4, a generous addition of the phenolphthalein indicator makes the initial pink readily detectable.

131. Temperature Effects on Solution Volumes

a. Within the range of ordinary laboratory temperatures, a temperature shift of 5° C. causes a volume change of about 1 part per 1,000 (for exact volume changes of water, or solutions not over 0.1 *N* in concentration, see table VII). It is desirable to prepare standard solutions at about the average laboratory temperature at which they will be used. Then laboratory deviations from this temperature will ordinarily cause errors of less than 1 part per 1,000. In titration of one standard solution against another, room temperature does not influence the results, because the percentage effects of its changes are the same on both solutions.

b. Volume effects of temperature change are much less on glassware than on water solutions; within the range of room temperature, the effects on glassware may usually be neglected. With Pyrex glass the volume changes per 1° C. change in temperature is only 1 part in 100,000; with ordinary glass, 2 or 3 parts per 100,000.

132. Adjustment of Standard Solutions

In preparing a solution of secondary standard, it is convenient to make the concentration at first somewhat more than the exact normality desired. The preliminary solution is then standardized, and is diluted to bring it exactly to the desired normality. If the volume of the preliminary solution left after standardizing it, is *V*

ml, and its normality factor is F , the solution is diluted to $F \times V$ ml to bring the factor down to 1.000. For example, if the preliminary factor is found to be 1.020, and the volume of solution is 950 ml, this solution is diluted to $1.020 \times 950 = 969$ ml by adding 19 ml of water, to make the final factor 1.000.

133. Standard Hydrochloric Acid Solutions From Constant-boiling HCl Solution

(Hulett and Bonner, J. Am. Ch. Soc., 31, 390 (1909))

a. GENERAL. When a hydrochloric acid solution of approximately 6 *N* concentration is distilled, the acid in the undistilled portion approaches a constant concentration, which depends on the barometric pressure at the time of distillation. If boiling is continued, water and hydrochloric acid, then distill off in constant proportions definable to 1 part in 10,000. The distillate then obtained is the "constant-boiling" solution.

Table XII. Constant-boiling Hydrochloric Acid Solution (Hulett and Bonner)*

Barometric pressure at time of distillation (mm of mercury)	HCl concentration percent by weight (gm HCl per 100 gm solution)	E=equivalent Weight of solution †
610-----	20.585	176.99
620-----	20.560	177.19
630-----	20.532	177.43
640-----	20.504	177.67
650-----	20.471	179.94
660-----	20.438	178.24
670-----	20.417	178.43
680-----	20.396	178.62
690-----	20.375	178.37
700-----	20.354	179.00
710-----	20.333	179.19
720-----	20.312	179.38
730-----	20.293	179.56
740-----	20.296	179.77
750-----	20.245	179.98
760-----	20.221	180.19
770-----	20.197	180.41
780-----	20.173	180.62

* Values for pressures 730 to 780 mm corrected by Foulk and Hollingsworth (J. Am. Ch. Soc., 45, 1220 (1923)); values for pressures 610 to 660 mm added by Bonner and Branting (J. Am. Ch. Soc., 48, 3093 (1926)); values for pressures 670 to 720 mm interpolated.

† E=gm of solution required to make 1 liter of 1 *N* HCl solution.

0.1 E=gm of solution required to make 1 liter of 0.1 *N* HCl solution.

† Also: E=mg of solution equivalent to 1 ml of 1 *N* HCl solution.

0.1 E=mg of solution equivalent to 1 ml of 0.1 *N* HCl solution.

The weights under E are for the solution weighed in air. If weighed in vacuum the weights would be about 1 part per 1,000 greater.

b. PREPARATION OF CONSTANT-BOILING HCl SOLUTION. To concentrated hydrochloric acid (specific gravity 1.19 to 1.20), add an equal volume of water. Bring the solution to a density of 1.096 to 1.098. (If a hydrometer is not available, weigh 10 ml, delivered from a calibrated pipette into a weighing bottle, to determine the density.) Place 500 to 2,000 ml in a distilling flask of capacity 1.3 to 1.5 times the volume of solution. In the flask place 10 1-inch lengths of Pyrex glass tubing of 1 or 2 mm diameter, to promote smooth boiling and prevent overheating. Protect the sides of the flask with asbestos, so that hot gases from the flame will not overheat the vapor space. Use a straight tube condenser. Distill away three-fourths of the liquid at such a rate that about 2 hours are required. Save this distillate as starting material for a second lot. The remaining one-fourth has the composition given in table XII. Of this quarter, distill three-fourths and collect the distillate; during collection keep the end of the condenser inserted into the receiving flask and near, but not dipping into, the liquid in the receiver. Read the barometer at the beginning and the end of the distillation of this last portion; average the readings and correct the average barometer reading for the room temperature. (With sufficient accuracy 2 mm may be subtracted as the correction at 15° to 20°, 3 mm at 20° to 27°, 4 mm above 27°.) The distillate is stored in a glass-stoppered bottle with a vaselined stopper. It keeps indefinitely. The number of grams of solution that contain 1 gram-equivalent of HCl is indicated by the third column of table XII. The bottle is labeled with this number, and with the barometric pressure at which the acid was distilled.

c. HYDROCHLORIC ACID SOLUTION, 0.1 *N*. Of the constant-boiling acid 16.4 ml (approximately 18 gm) is measured into a 50 ml Erlenmeyer flask which has previously been weighed to 1 mg. More of the constant-boiling acid is added to or withdrawn from the flask with a medicine dropper until the weight of the solution in the flask is within 5 mg of the amount required for 1 liter of 0.1 normal solution (0.1 *E.*, table XII). The acid is then at once diluted by adding nearly enough water to fill the 50 ml flask, and the 50 ml flask is filled five times with water and emptied into the 1 liter flask. The liter flask is then filled about three-quarters full by addition of water, with which the acid is mixed by whirling. The solution is then diluted to the mark, and is mixed by inverting the flask.

If kept in an ice box the 0.1 *N* solution is stable. If exposed to ordinary laboratory conditions of light and temperature, however, the acid may weaken at the rate of 1 or 2 percent per year, apparently because of reaction with atmospheric oxygen. For precise use, the 0.1 *N* HCl should either be prepared fresh once in 2

months, or should be kept in an ice box and brought to room temperature only when being used.

d. **HYDROCHLORIC ACID, 1.0 N.** To prepare 250 ml of 1 N solution, 41 ml of the constant-boiling acid is placed in a weighed 50 ml Erlenmeyer flask. With a medicine dropper, solution is added to or withdrawn from that in the flask until the weight of solution is within ± 10 mg of the weight containing 0.25 gram-equivalent of HCl (0.25 *E*, table XII). The solution is then poured into a 250 ml volumetric flask. The Erlenmeyer flask is washed repeatedly with water until the washings nearly fill the 250-ml flask, the added washings being mixed by whirling with the solutions in the flask. The volume is finally made up to 250-ml with water.

134. Standardization of Solutions of Other Acids by Comparison With Standard HCl Solutions

With a transfer pipette measure into a 125-ml Erlenmeyer flask 20 or 25 ml of HCl solution of the normality for which the other acid solution is designed (for example, 0.1 or 1.0 N). With the same pipette the same volume of the other acid solution is measured into another 100-ml Erlenmeyer flask. The two acid solutions are titrated with standard sodium hydroxide solution delivered from the same burette, which is filled to the zero point for each titration. The normality factor of the unknown acid solution is calculated as:

$$\text{Factor} = \frac{\text{ml of NaOH solution used for unknown acid}}{\text{ml of NaOH used for standard HCl}}$$

This procedure eliminates the effects of errors of calibration of different pieces of apparatus and of error from inaccurate standardization of the NaOH solution.

135. Standard Sulfuric Acid Solutions ($\text{H}_2\text{SO}_4=99.08$)

a. **SULFURIC ACID, 1.0 N.** This solution is half molar. Prepare a preliminary solution of somewhat more than normal concentration by adding slowly, with stirring, 31 ml of concentrated sulfuric acid (sp. g. 1.84) to 1 liter of water. Cool to room temperature and standardize by comparison with 1.0 N hydrochloric acid solution, as described in paragraph 134. Use the preliminary factor thus obtained, and dilute to factor 1.000, as described in paragraph 132. The diluted solution is checked by comparing again with 1.0 N hydrochloric acid solution. If 1.0 N HCl from the constant boiling solution is not available, the preliminary and final factors of the 1.0 N H_2SO_4 may be determined by titration against 1.0 N NaOH that has been standardized against weighed portions

of constant boiling HCl solution or of potassium acid phthalate, as described in paragraph 136.

b. 0.1 *N* AND OTHER DILUTE SULFURIC ACID SOLUTIONS. These are prepared by dilution of the 1.0 *N* solution, and are checked by comparison with standard hydrochloric acid solutions (par. 134), or by titration with standard sodium hydroxide solutions.

136. Standard Sodium Hydroxide Solutions ($\text{NaOH}=40.01$)

a. PREPARATION OF CONCENTRATED SOLUTION OF MINIMAL CARBONATE CONTENT. Dissolve 100 to 5,000 gm of pure sodium hydroxide in an equal weight of water in a Pyrex flask or an earthenware jar. The solution becomes hot, and would be likely to break a glass vessel other than Pyrex. Cool to room temperature and transfer to a paraffin-lined bottle (see below) or to a cylinder closed with a paraffin-lined stopper. Sodium carbonate (Na_2CO_3) is practically insoluble in the concentrated sodium hydroxide, and settles to the bottom in a few days. The clear solution is 18 to 20 *N*.

If much of this solution is used, it is desirable to begin with at least 2,000 gm of sodium hydroxide. The clear solution is transferred for storage to a 4-liter paraffin-lined aspirator bottle, which is protected by a soda-lime tube in the top and has inserted in the bottom outlet, a short glass tube, which bends downward and passes into a heavy-walled rubber tube, 7 or 8 cm long, that is closed by a screw-clamp. The rubber tube connects with a glass capillary tube of 2 mm bore, 5 to 7 mm outer diameter, and about 10 cm length, which serves as outlet. A rubber stopper is fitted over the glass capillary a few cm above the tip. A heavy-walled test tube fitted over this stopper prevents atmospheric CO_2 from forming a crust of Na_2CO_3 about the capillary outlet.

From 5 to 5.7 ml of the alkali solution diluted to 1 liter makes a 0.1 *N* solution. The amount required is found to within 0.1 ml by trial and is marked on the bottle.

b. 1.0 *N* NaOH SOLUTION.

- (1) *Preliminary solution.* A preliminary solution somewhat stronger than 1.0 *N* is first made and standardized, and is then diluted to exactly 1.0 *N*. In a 1-liter volumetric flask place about 800 ml of water and add somewhat more than enough of the concentrated NaOH solution to make 1 liter of normal solution; usually 60 ml of the concentrated solution suffices. Stopper the flask and mix the solutions by rotating. The solution warms. Cool it to room temperature, dilute to the mark with water, and

mix by repeated inversion. Standardize by either of the following methods.

- (2) *Standardization against weighed portions of constant-boiling HCl solution.* Weigh a 50-ml Erlenmeyer flask to within 1 mg. With a graduated 5-ml pipette measure into the flask 3.65 ml (approximately 4 gm) of the constant-boiling solution. Weigh the flask plus the solution to within 1 mg. Then at once add 10 ml of water, and stopper the flask until ready to titrate. Prepare three flasks thus with weighed portions of constant-boiling HCl. Titrate the solutions with the preliminary NaOH solution delivered from a 25-ml burette. The 1.0 normal factor of the NaOH solution is calculated as:

$$1.0 \text{ } N \text{ factor} = \frac{\text{mg constant boiling HCl solution}}{E \times \text{ml NaOH solution}}$$

E is the equivalent weight of the constant-boiling HCl solution indicated by table XII. (For the solution distilled at 760 mm pressure $E = 180.19$.)

- (3) *Standardization against acid potassium phthalate.* Bureau of Standards phthalate must be used. Weigh three portions of 4 to 4.5 gm each of the phthalate and transfer each to a 250-ml Pyrex Erlenmeyer flask. Add 100 ml of water to one of the flasks. Holding the flask in a pair of tongs or a test-tube holder, heat over a free flame until the phthalate is dissolved and the solution begins to boil. Then at once add 10 drops of 1 percent phenolphthalein solution and titrate with the NaOH solution from a 25-ml burette. Repeat with the other two flasks. The 1.0 N factor of the NaOH solution is calculated as:

$$1.0 \text{ } N \text{ factor} = \frac{\text{mg phthalate}}{204.2 \times \text{ml NaOH solution}}$$

- (4) *Dilution to exact normality.* The preliminary solution left after standardizing is diluted to 1 N as described in paragraph 132. The accuracy of the final solution is checked by standardization against either constant-boiling HCl or phthalate as described above.

c. 0.1 N NaOH SOLUTION.

(1) *Preparation.*

- (a) *From 1.0 N solution.* Place 800 ml of water in a 1-liter volumetric flask. Measure 100 ml of the exact 1.000 N NaOH in a 100 ml volumetric flask and pour into the liter flask. Rinse adherent alkali in the 100 ml flask into the liter flask with several portions of water, add

enough additional water to make the volume up to 1 liter, stopper at once, and mix by inversion.

- (b) *From concentrated NaOH solution.* Make a preliminary solution as described for 1 *N* solution, except that about 6.0 instead of 60 ml of the concentrated solution is used. Standardize the solution, as described below, then dilute to exactly 0.1 *N* concentration, as described in paragraph 132, and standardize again.
- (2) *Standardization against weighed portions of constant-boiling HCl solution.*
- (a) Weigh a 50-ml Erlenmeyer flask to within 0.1 mg. For weighing with such accuracy the flask must be at the temperature of the balance, and must be handled with tongs, not by the hands, which might warm it enough to affect the weight. After the weighing, place 400 mg of additional weights on the right-hand pan. Remove the flask from the balance with tongs, and run into the bottom 0.365 ml of the constant-boiling HCl measured from a 1-ml graduated pipette. This will give 400 ± 5 mg. The flask is replaced on the balance and weighed again to within 0.1 mg. Then 10 ml of water is at once added, and the flask is stoppered until titrated.
- (b) The constant-boiling HCl solution volatilizes so slowly from the open flask during weighing that about 10 minutes are required for the loss to amount to 1 part per 1,000. The weighing of the flask plus acid, as described, with the weights to within 5 mg already on the pan, takes only 1 or 2 minutes, so that loss by volatilization is negligible, and use of a stoppered flask for the weighing is unnecessary.
- (c) The HCl solution in the flask is titrated with the 0.1 *N* NaOH from a 25-ml burette. The 0.1 *N* factor of the NaOH solution is calculated as:

$$0.1 \text{ } N \text{ factor} = \frac{\text{mg constant-boiling HCl solution}}{0.1 \text{ } E \times \text{ml NaOH solution}}$$

E is the equivalent weight of the constant boiling solution indicated by table XII. (For the solution distilled at 760-mm pressure $0.1 \text{ } E = 18.02$).

- (3) *Standardization against weighed portions of acid potassium phthalate.* Bureau of Standards phthalate must be used. Weigh three portions of 400 to 450 mg each to within 0.1 mg and transfer to 50 ml Erlenmeyer flasks. Add 10 ml of water to each, heat each in turn until the

solution begins to boil, add 5 drops of 1 percent phenolphthalein solution and titrate the hot solution with the NaOH solution from a 25-ml burette. The end-point is the first pink color that pervades the entire solution for 15 seconds.

$$0.1 \text{ N factor} = \frac{\text{mg phthalate}}{20.42 \times \text{ml NaOH solution}}$$

d. 0.01 N NaOH SOLUTION. Alkali solutions of this order of dilution are prepared by exact dilution of standardized 0.1 N solution. They are so sensitive to change by contact with air or glass that it is often preferable to prepare them daily, rather than to use the necessary precautions to keep them.

e. STORAGE OF STANDARD ALKALI SOLUTIONS. Standard solutions of alkali should be *protected against absorption of carbon dioxide* from the atmosphere by requiring all air that enters the stock bottle to pass through tubes containing granules of soda lime. To prevent fine particles of the soda lime from being carried in by an air current, there should be a firmly packed plug of cotton, 2 or 3 cm thick, between the soda lime and the solution. As an added precaution, bend the narrow stem of the tube, so that the barrel containing the soda-lime hangs down over the shoulder of the bottle.

Alkaline solutions act on glass and thereby the titer is changed. If the glass is relatively resistant, such as Pyrex, the action is usually so slow that 1.0 N or 0.1 N solutions will change by less than 1 part per 1,000 in 1 month, but more dilute solutions of alkali change more rapidly in contact with glass, even if the latter is Pyrex.

To protect a standard solution of alkali against action on glass, the inside of the container should be coated with paraffin of melting point not less than 55° C. Before paraffining a bottle, thoroughly clean and dry it. Warm the bottle to a temperature only slightly above the melting point of the paraffin and pour in sufficient melted paraffin to make a thick coat. While the bottle cools roll it gently to distribute the coat evenly, and just before the last portion of paraffin solidifies, stand the bottle upright to allow the excess of paraffin to collect on the bottom and to form a substantial layer. In warm climates even paraffins with the highest melting points may soften and give way. Cracks in the paraffin should be watched for, lest there be a false sense of security.

For room temperatures above 35° C., wax of higher melting point must be used. The mineral wax (Cerasin) can be used.

f. EFFECTS OF CO₂ AND DISSOLVED GLASS ON STANDARD NaOH

SOLUTIONS.

- (1) *Absorption of CO_2* has the effect of neutralizing some of the alkali, and thereby *lowering the normality factor* of the solution. The effect is greatest when an end-point like that of phenolphthalein, above pH 8, is used. Titration to this end-point leaves the CO_2 practically all in the form of NaHCO_3 , so that each mole of CO_2 present decreases the alkali titer by one equivalent. When a lower pH is used as end-point, the CO_2 has less effect; at pH 6 the effect is only half as great, and at pH below 5 it is slight, because nearly all the NaHCO_3 , in titration against HCl, is changed to H_2CO_3 and NaCl.
- (2) *Solution of silicate from glass increases the normality factor* of the standard solution, especially when the end-point is below about pH 7, and *decreases the sharpness of end-points*, both above and below pH 7. As an example, a 0.1 N NaOH solution, which when fresh had the factor 1.000 determined by titration against HCl with either phenolphthalein or methyl red (pH about 5), after keeping 2 years in a Pyrex glass bottle protected from atmospheric CO_2 , showed its factor increased to 1.001 when titrated with phenolphthalein, and to 1.004 with methyl red. Decrease in sharpness of end-point was shown by the fact that, whereas when 20 ml of 0.1 N HCl was titrated with the fresh 0.1 N NaOH, with phenolphthalein as indicator to the first permanent pink color, a single additional drop (0.05 ml) changed the color to deep red, 0.35 ml of the 2-year solution was required to produce the same change.

g. DETECTION OF CO_2 AND SILICATES IN STANDARD NaOH.

- (1) *By difference between normality factors determined with methyl red and with phenolphthalein.* The factor determined with methyl red is higher than that determined with phenolphthalein, if either glass or CO_2 is dissolved. Of glass the chief effect is to raise the factor determined with methyl red; of CO_2 the chief effect is to lower the factor determined with phenolphthalein.
- (2) *By decrease in sharpness of end-points*, as exemplified under f above.
- (3) *By precipitation with barium chloride.* Add a few drops of 5-percent barium chloride solution to 10 ml of the alkali solution. An opalescence or precipitate will form if either carbonate or silicate is present in considerable

amount. If the precipitate dissolves on acidification with acetic acid, it is carbonate.

137. Potassium Iodate Solution, 0.1N ($\text{KIO}_3=214.02$)

Potassium iodate standards are used chiefly in titrations with thiosulfate. (See pars. 125 and 126.) For this titration, the equivalent weight of potassium iodate is one-sixth of the molecular weight, or 35.67. Weigh 3.567 gm of pure potassium iodate, transfer to a 1-liter flask, and make up to the mark with distilled water. Potassium iodate is a particularly useful standard, because the salt can be obtained in good purity, and because the solution lasts indefinitely without changing. It is a primary standard, and its preparation by weight gives it more accuracy than can be obtained by standardizing it against a reducing agent; hence, such standardization is unnecessary.

138. Potassium Dichromate Solution, 0.1 N ($\text{K}_2\text{Cr}_2\text{O}_7=294.21$)

Potassium dichromate is a primary standard, stable both in solid form and in solution. It can be purified by recrystallization from water, and can be dried at temperatures up to 200°C . without decomposition. It is used in titrations in which Cr^{+++++} is reduced to Cr^{+++} . (See par. 126.) Hence, the equivalent weight is one-sixth of the molecular weight. To prepare a 0.1 N solution, dissolve 4.904 gm of potassium dichromate in water in a 1-liter flask, and dilute to the mark.

139. Potassium Permanganate Solution, 0.1 N ($\text{KMnO}_4=158.026$)

a. PREPARATION. A liter of this solution should contain 1/50 gm. molecule, or 3.1605 gm, of potassium permanganate. (See par. 126.) The solution can be made accurately by weight from the pure crystals. It tends to become weaker, however, after standing. Hence it is desirable to let it stand for a week before it is used, and then to standardize it against sodium oxalate.

b. STANDARDIZATION WITH WEIGHED SODIUM OXALATE. Weigh 250 to 300 mg of pure sodium oxalate to 0.1 mg, and dissolve it in 200 to 250 ml of hot water in a 400 ml beaker. The water should be at a temperature of 80° to 90°C ., since the titration must be completed at a temperature above 60°C . Add 10 ml of 1:1 sulfuric acid (1 part of concentrated acid plus 1 part of water), and titrate at once with the 0.1 N potassium permanganate solution, stirring the liquid vigorously and continuously. A thermometer makes a convenient stirring rod. The permanganate must not be added more rapidly than 10 to 15 ml per minute, and the last 0.5

to 1 ml should be added dropwise, allowing each drop to be decolorized before the next is added. The excess of permanganate necessary to produce the end-point color should be estimated by matching the end-point color in another beaker containing the same amount of hot water and sulfuric acid as in the titration. Deduct this amount from the titration.

The normality factor is calculated as follows, 67 mg being the milliequivalent of the oxalate (par. 142) :

$$0.1 \text{ N factor} = \frac{\text{mg oxalate}}{6.7 \times \text{ml KMnO}_4 \text{ solution}}$$

If the solution is near 0.1 *N* strength, it is generally used as it is, and the 0.1 *N* equivalent in any titration is calculated by multiplying the ml used by the factor determined in the standardization.

140. Iodine Solution, 0.1 *N* (I=126.92)

a. PREPARATION. Iodine can be weighed, with precautions to prevent loss by volatilization, and used as a primary standard. The usual practice, however, is to prepare an approximate solution, and standardize it with thiosulfate solution. For each liter of solution desired, weigh 13 gm of iodine, instead of the 12.69 gm actually required, into a weighing bottle. Dissolve about 30 gm of pure potassium iodide in about 250 ml of water in a beaker. Transfer the iodine from the weighing bottle to a 1-liter volumetric flask, and wash out any adhering iodine crystals with the iodide solution. Pour the rest of the KI solution into the liter flask. Mix the contents of the flask until the iodine is completely dissolved and then dilute to volume.

b. STANDARDIZATION WITH 0.1 *N* THIOSULFATE SOLUTION. Standardize by titrating 20 ml with 0.1 *N* thiosulfate solution, with or without starch indicator. The normality factor is calculated as:

$$0.1 \text{ N factor} = \frac{\text{ml of 0.1 N thiosulfate}}{\text{ml of iodine solution}}$$

141. Sodium Thiosulfate Solution, 0.1 *N* (Na₂S₂O₃ · 5H₂O=248.19)

a. PREPARATION. The molecular weight, 248.19, is the same as the equivalent weight when the thiosulfate is titrated against iodine. (See par. 126.) Dissolve 24.82 gm of crystalline sodium thiosulfate in water in a 1-liter volumetric flask. Add 3 gm of borax to serve as a preservative. Dilute to 1 liter, and mix. The solution should be restandardized every 2 months, since it is not entirely stable.

b. STANDARDIZATION WITH WEIGHED POTASSIUM DICHROMATE. Weigh 200 to 230 mg potassium dichromate to 0.1 mg and dissolve in 100 ml of water in a 250 ml glass-stoppered Erlenmeyer flask. Add 20 ml of 1.0 *N* hydrochloric acid and 2 gm of potassium iodide. Stopper the flask to prevent loss of volatile iodine, and let it stand in the dark for 10 minutes while the dichromate reacts with the iodide. Then titrate at once with the thiosulfate from a 50-ml burette, adding 2 or 3 drops of 1 percent starch solution when the iodine color becomes faint. According to the reactions shown in paragraph 126 a gram equivalent of potassium dichromate is one-sixth of a gram molecule, or $294.21 \div 6 = 49.04$ gm. Hence, the normality factor is calculated as:

$$0.1 \text{ } N \text{ factor} = \frac{\text{mg K}_2\text{Cr}_2\text{O}_7}{4.904 \times \text{ml thiosulfate}}$$

c. STANDARDIZATION WITH WEIGHED POTASSIUM IODATE. The molecular weight of potassium iodate (KIO_3) is 214.02. The equivalent weight in this titration is one-sixth of this, or 35.67. (See par. 126.)

From 140 to 160 mg of purest reagent-grade potassium iodate is weighed to within 0.1 mg and transferred to a 250-ml flask. The iodate is dissolved in 50 ml of water, and 2 to 3 gm of potassium iodide or sodium iodide are added, followed by 10 to 15 ml of 1.0 *N* sulfuric acid. The iodine set free is *at once* titrated with the 0.1 *N* thiosulfate from a 50 ml burette, until the iodine color fades to a light yellow. Then add 3 drops of starch indicator solution, and continue the titration until there is no color. It is preferable not to add the starch sooner, because the gradual decrease in yellow iodine color enables one to titrate without hesitation nearly to the end. The normality factor is calculated as:

$$0.1 \text{ } N \text{ factor} = \frac{\text{mg KIO}_3}{3.567 \times \text{ml thiosulfate}}$$

d. STANDARDIZATION WITH 0.1 *N* POTASSIUM IODATE SOLUTION. Twenty ml of 0.1 *N* potassium iodate (par. 137) are delivered into a 125-ml flask. Add 5 to 10 ml of 1.0 *N* sulfuric acid, and about 1 gm of potassium iodide or sodium iodide. Titrate *at once* with the 0.1 *N* thiosulfate from a 25 ml burette until the iodine color fades to light yellow. Then add 2 drops of starch indicator solution, and continue titration until colorless. The normality factor is calculated as follows:

$$0.1 \text{ } N \text{ factor} = \frac{\text{ml } 0.1 \text{ } N \text{ KIO}_3}{\text{ml thiosulfate}}$$

142. Sodium Oxalate Solution, 0.1 N ($\text{Na}_2\text{C}_2\text{O}_4=134.01$)

The oxalate is a primary standard, and is used to standardize permanganate and ceric sulfate solutions. It can be dried at temperatures up to 130°C . The reaction with permanganate (par. 126c) in acid solution is such that the equivalent weight is half the molecular weight. To make a 0.1 N solution, 6.700 gm of sodium oxalate are dissolved in water and diluted to 1 liter.

143. Starch Indicator Solution

a. Make a 1 percent solution by dissolving 1 gm of high-grade soluble starch in 50 ml of boiling water and dilute to 100 ml. Starch solutions without preservatives become moldy and deteriorate after a few days. This deterioration may be prevented, or greatly retarded, by adding 1 gm of salicylic acid per liter to the water used in preparing the solution, or by saturating the fresh starch solution with sodium chloride.

b. Another method of preparing starch solution uses zinc chloride as preservative. Triturate 6 gm of soluble starch with cold water to a thin paste, and rinse into a liter of boiling water. To this is added 50 ml of a solution containing 6 gm of zinc chloride. The starch solution is distributed in small, tightly corked bottles.

144. Solution Concentrations Defined by Percentage Content

Unless defined, the term "percent" is ambiguous. It may mean parts of solute per 100 ml of solvent, or per 100 gm of solvent, or per 100 ml of solution, or per 100 gm of solution. In this manual, percent concentration of solutions indicates *grams of solute per 100 ml of solution*, unless otherwise stated.

Note. See the section "Volumetric Solutions" in the *U. S. Pharmacopoeia*, to Van Nostrand's Chemical Annual, or to "Volumetric Primary Standards" in *The Handbook of Chemistry and Physics* of the Chemical Rubber Company, etc., for tables of equivalents of 1.0 N and 0.1 N solutions.

145. Atomic Weights.

A partial list of atomic weights follows for convenience and use in making any necessary chemical calculations which may be required in the use of chemical methods outlined in this manual. Only those atomic weights which are believed to be of most value are included.

Name	Atomic Weight	Name	Atomic Weight
Aluminum -----	26.97	Hydrogen -----	1.008
Antimony, stibium -----	121.76	Iodine -----	126.92
Arsenic -----	74.91	Iron, ferrum -----	55.85
Barium -----	137.36	Lead, plumbum -----	207.21
Bismuth -----	209.00	Magnesium -----	24.32
Bromine -----	79.916	Manganese -----	54.93
Calcium -----	40.08	Mercury, hydrargyrum ---	200.61
Carbon -----	12.01	Nitrogen -----	14.008
Chlorine -----	35.457	Oxygen -----	16.000
Chromium -----	52.01	Phosphorus -----	30.98
Cobalt -----	58.94	Silver, argentum -----	107.880
Copper -----	63.57	Sodium, natrium -----	22.997
Gold, aurum -----	197.2	Sulfur -----	32.06

Section VIII. COLORIMETRY

146. General

a. COLORIMETRY PRINCIPLES.

- (1) Colorimetry, in the limited sense that the term has acquired in analytical chemistry, consists in passing equal beams of light through two solutions of a colored solute, and regulating either the concentrations or the depths of the transmitting layers until the absorption of visible light is the same in both solutions, as indicated by an exact match between the fields of transmitted light. From the relative volumes or layer depths the relative amounts or concentrations of colored solute in the two solutions are calculated.
- (2) Every substance when in solution absorbs radiant energy (of some part of the spectrum) either within a part of the spectrum that is visible (light) or in the ultra-violet or infra-red zones. When the absorption is within a limited part of the visible spectrum the solution appears to be colored to a person with normal vision. There are several ways of using the phenomenon of absorption for analytical purposes. One is to prepare a graded series of standard solutions with which the solution under test is compared by viewing through equal depths against a background of uniform illumination. This is illustrated in the colorimetric method of determining pH values. (See par. 122.) Other simple methods are described in this section. If the so-called "transmittance" is to be measured and related to the concentration of an absorb-

ing substance, it is necessary to use a photometer, as described in section IX of this chapter.

- (3) The relative visibility of light of ordinary illumination is maximal for yellow-green light of wave length 550 to 560 millimicrons and falls off rapidly with increase and decrease of wave length. At 420 and at 700 millimicrons the visibility is only about 1/250th of the maximum. Owing to the limitations of visibility the use of the eye is restricted, and if the absorption occurs only in the infra-red or ultraviolet zones a photoelectric cell must be used, as described in section IX of this chapter.

b. ANALYTICAL COLORIMETER. This is a device for adjusting the volumes or layer depths of the compared solutions, and for matching the color fields. In accurate colorimeters there is an optical system to assist in matching the fields of the two transmitted beams.

c. BASIS OF CALCULATION. The relative amounts of colored solute in the two solutions are calculated on the assumption that transmissions of light equal with regard to all wave lengths occur when the two beams are subjected to absorption by the same amounts of colored solute, whether these amounts are dispersed through transmitting columns of equal or of different lengths. If one of the solutions is a known standard, the amount of colored solute in the other solution can be calculated by formulas based on this assumption.

d. SOURCE OF LIGHT. Under the conditions of equal transmission of the two beams postulated in *c* above, colorimeters are not subject to the restriction of photometers, that the incident light must be monochromatic for strict accuracy. For a colorimeter one may use white light or any desirable colored light, with or without a color filter.

e. STANDARD SOLUTIONS. When white light is used it is generally necessary to prepare the standard solution with the same colored solute as the unknown in order to obtain accurate results; exact matches are difficult to obtain with "artificial" standards prepared from colored substances other than those to be determined.

In some analyses, however, it is possible to use such artificial standard solutions, or even colored glass standards, approximating the color of the unknown. The stability or ease of preparation of such standards may in some cases be more important than exact matching. Matching may be improved by the use of colored light filters. (See par. 148.)

147. Dilution Colorimeters

a. In this type of colorimeter, exemplified by the Sahli hemoglobinometer, the relative *concentrations are varied* in the two compared solutions by diluting one of the solutions until the colors, viewed through equally thick layers of the two, match. The concentrations are then equal, and the relative amounts of the colored solute in the two solutions are proportional to their volumes. Letting a represent the amount (not the concentration) of colored solute in the standard solution, x the amount in the unknown, and V_s and V_u represent the volumes of standard and unknown solutions respectively, the following proportion holds:

$$x : a = V_u : V_s, \quad (1)$$

whence the amount of solute in the unknown solution is calculated as follows:

$$x = a \frac{V_u}{V_s}. \quad (2)$$

b. Dilution colorimetry can be carried out with the simplest of apparatus. An apparatus capable of 5 or 10 percent accuracy can be improvised from two cylinders of equal bore viewed against a window or a white or colored background. Besides its simplicity, the dilution colorimeter has the advantage that the proportion in equation (1) is valid even if the solution does not obey Beer's law (sec. IX of this ch.), because equal concentrations are present in both standard and unknown. Also, if an interfering colored solute is present (such as the alkaline picrate in creatinine determinations), its effect can be eliminated by adding the interfering solute to the solvent used in the diluting procedure.

148. Duboscq Type of Colorimeter

a. PRINCIPLE. In this type of colorimeter two compared solutions are permitted to retain their original concentrations, and equality of transmission is obtained by *varying the length* of one of the two solution layers through which the light is transmitted. Usually the layer of standard solution is set at 15 or 20 mm, and the layer of the unknown is varied until the two fields of transmitted light match. The lengths of the two transmitting layers of solution are then inversely proportional to the concentrations of colored solute.

b. CALCULATIONS.

- (1) Letting C_u and C_s represent the concentrations in the unknown and standard solutions respectively, while U and

S represent the length of the transmitting layers of the unknown and standard solutions respectively, the proportion holds:

$$C_u : C_s = S : U, \quad (3)$$

whence the concentration of solute in the unknown solution is calculated as:

$$C_u = C_s \frac{S}{U}. \quad (4)$$

- (2) When the unknown solution is prepared by filtration or otherwise from blood or urine in such a way that one volume of blood or urine is represented by d volumes (dilution) of the final solution, the concentration of the analyzed solute in the original blood or urine is

$$d \times C_u, \text{ or } d \times C_s \times \frac{S}{U}.$$

The general calculation formula used in clinical analyses with the Duboscq type of colorimeter is therefore:

$$\text{Concentration of substance in blood or urine} = d \times C_s \times \frac{S}{U}. \quad (5)$$

In determination of a given substance d and C_s are often the same in all or most analyses, and when constant they can be combined into a single factor, $d \times C_s$.

Example. In blood sugar analyses 10 ml of the Folin-Wu filtrate used represents 1 ml of blood; hence, $d = 10$. If the standard contains 20 mg of glucose per 100 ml, C_s is 20, and the calculation is as follows:

$$\text{Mg sugar per 100 ml blood} = 10 \times 20 \times \frac{S}{U} = 200 \times \frac{S}{U}.$$

c. CONDITIONS UNDER WHICH CALCULATION FORMULAS DO NOT HOLD.

- (1) When the light-transmitting property of the analyzed solute varies with concentration and thereby fails to obey Beer's law (sec. IX of this ch.), equations (3), (4), and (5) do not hold. To produce this inconvenient anomaly, however, three conditions must occur together:
 - (a) The analyzed substance must exist in two or more states in the solution. (Part may be ionized, part not; part may be loosely bound to proteins, part not so bound; or the substance may be in different states of aggregation).

- (b) The substance in the different states must transmit light differently (different colors or different proportions of total light energy).
- (c) The proportions of the substance in the different states must vary with the concentration of the substance.

When these conditions occur together a layer of solution 2 cm long with c concentration of the colored solute will *not* show light transmission identical with the transmission of a layer 1 cm long and of $2c$ concentrations, as required by equations (3, and (4).

- (2) *The equations also do not hold when solutes, other than the solute to be analyzed, are present that absorb light in the visible portions of the spectrum.* Such substances obviously make it impossible to compare the solution with a standard that contains no light-absorbing material except the substance to be analyzed.

d. USE OF EMPIRICAL CURVES WHEN CONDITIONS INVALIDATE CALCULATION FORMULAS.

- (1) *When the light-transmitting property of the analyzed solute varies with concentration* (see above), one may

S

prepare empirical curves of C_u versus the ratio $\frac{S}{U}$. In

preparing such a curve the unknown is replaced by varying concentrated standard solutions of the substance to be determined; a single standard in the left cup is compared with a series of varying standards in the right cup.

S

Graphic interpolation of $\frac{S}{U}$ values observed in analyses

of unknown solutions is used to calculate concentrations.

- (2) *When a light-absorbing substance other than the analyzed solute is present in constant concentration*, calculations can be made from an empirical curve prepared as above, but with the known concentration of the interfering substance present in the standard solutions in both cups. Use in this manner of an empirical curve increases the accuracy of creatinine determinations, in which a constant amount of colored picrate is always present.

e. COLOR FILTERS. The effects of interfering colored material may be minimized by a color filter which absorbs light of wave lengths transmitted in common by both the determined substance and the interfering material, and which transmits light of wave lengths absorbed only by the determined substance. Such use of a

filter presupposes the existence of a range of visible wave lengths absorbed by the analyzed substance, and not by the interfering material. If a colored substance other than the determined substance is used as a standard (for example, dichromate for creatinine), the differences in color shade, which are always present, can be minimized by use of a filter on the above principles. Filters are used in the microkjeldahl nitrogen, urea, and creatinine analyses described in this manual.

f. TECHNIC FOR USE OF DUBOSCQ COLORIMETER.

- (1) *Cleaning cups and plungers.* The cups and plungers must be scrupulously clean. If they are not, rinse them with distilled water and dry with filter paper. Inspect the windows at the bottoms of the plungers and cups and, if any film is present, polish with lens paper or with soft filter paper.
- (2) *Zero points.* The plungers are carefully brought into contact with the bottoms of the cups. Both scales should read zero. If either one does not, adjust it, if the colorimeter permits such adjustment. Otherwise readings must be corrected for the zero point error. For example, if the reading on the left scale is -0.2 mm, 0.2 mm must be added to all readings on this scale.
- (3) *Equality of light transmitted from both cups.* Place portions of the same standard solution, for the analysis to be done, in both cups, and bring the cups carefully up against the bottoms of the plungers in order to remove air bubbles trapped under the plungers. Then set both scales at 20 mm. The two fields should match. If they do not, adjust the angle of the mirror or the position of the source of light until equality is attained. Confirm the adjustment by making a series of readings. (See (5) below.) The average should be 20 mm. If it is not, re-adjust the light and repeat.
- (4) *Filling cups.* Leave the standard solution in the left cup. Empty the right cup, rinse the cup and plunger three times with small portions of the unknown solution, or if the amount of solution is too limited to provide for rinsing, wash the cup and plunger with water and then dry them with filter paper. Follow the same procedure whenever it is necessary to change the standard in the left cup. Remove any trapped bubbles as in (3) above. Never fill with so much solution that when the plunger is inserted the cup will overflow.
- (5) *Reading colorimeter.* Lower the right cup until the right field is definitely lighter than the left. Then raise it until

the right field is darker to about the same extent. Repeat this process of raising and lowering the right cup fairly rapidly two or three times and then step at the center of the range of travel where the fields appear to be of equal intensity. Note the reading and repeat the process. After the eye is adjusted to the colorimeter, three to five readings usually suffice for as much accuracy as can be obtained. If the first readings differ considerably from the later ones, disregard the first in calculating the average. (This applies only when the color is stable; in some determinations the color fades fairly rapidly in light.) If the eye tires, accuracy decreases. Raise the eyes frequently. Do not make an unnecessary number of repeated readings. When possible, use the colorimeter in a dimly lighted room with an artificial source of light for the instrument.

- (6) *Limits of difference between unknown and standard solutions.* For accuracy, the concentration of the standard should not be more than twice nor less than half the concentration of the unknown solution. If the standard is set at 15 mm, the reading of the unknown should not be less than 7.5 nor more than 30 mm; preferably it should be between 10 and 25 mm. When the unknown may vary over a wide range, prepare several standards so that one of them will approximate the unknown.
- (7) *Caring for colorimeter.* Keep solutions and reagents free from contact with the mirror (Nessler's solution is especially destructive to the silver backing.) Clean and dry the cups and plungers as soon as analyses are finished. When the colorimeter is not in use, cover it so that it will be protected from dust.

Section IX. PHOTOMETRY

149. Photometric Principles

In photometry the percentage of light that is transmitted through a colored or turbid solution serves to measure the concentration of the light-absorbing material. The transmittance does not need to be matched directly with that of a known standard solution. Consequently the photometer has an advantage over the colorimeter in that a fresh standard solution need not always be prepared for each series of analyses, although in some analyses fresh standards are required and in other analyses frequent checking with standards is desirable. A more significant ad-

vantage of the photometer is that it permits one, without complicating additions to the procedure, to correct for color or turbidity of the solvent or reagents. Furthermore the photometer lends itself to the use of a photoelectric cell in place of the human eye. With such cells, and especially designed photometers, it is possible to measure transmission of ultraviolet and infra-red rays outside the limits of visibility, and thereby to determine substances that absorb radiant energy in those ranges. Visible light covers the following approximate wave lengths, given in millimicrons: violet, 400 to 440; blue, 440 to 490; green, 490 to 560; yellow, 560 to 600; orange and red, 600 to 730.

150. Beer's Law

a. Beer's law is the basis of calculations in photometric analyses. It was developed from Bouguer's law, which states that layers of a given light-transmitting medium of equal thickness transmit equal fractions of the incident light that passes into them. The working of this law may be visualized as follows. Imagine a number of plates of translucent glass, each of which is 1 cm thick, and absorbs 0.7 and transmits 0.3 of the light that enters it. If two of these plates are placed in series, the first will transmit 0.3 of the incident light, and the second will transmit 0.3 of what passes through the first, or $0.3 \times 0.3 = 0.09$ of the original light. (Loss of reflected light is neglected in this illustration.) In general, if t represents the fraction of light (0.3 in the above example) transmitted by a 1-cm layer of glass or other transmitting medium, the fraction transmitted by a layer 1 cm long will be t^l .

b. In Beer's law, Bouguer's principle is applied to colored solutions, with the assumption that doubling or tripling the *concentration* of the colored solute has the same effect on the transmission as doubling or tripling the length of the transmitting layer. This assumption has in fact been found to hold for a large proportion of colored solutions. For such a solution, if the colored solute in a layer 1 cm long and of unit concentration transmits a fraction t of the incident light, then the solute in a 1-cm layer of c concentration will transmit the fraction t^c ; and in a layer of c concentration and l length the solute will transmit the fraction t^{lc} . This is Beer's law. It is formulated as:

$$T = t^{lc}, \quad (1)$$

where T represents the fraction of light transmitted by the colored solute.

c. In chemical analyses, T is measured as the ratio, I_s/I_b , in which I_s is the intensity of light transmitted through a cuvette containing the solution of colored solute, and I_b is the intensity of

light, from the same or an equal source, transmitted by a cuvette of equal size filled with a *blank solution*, in which the colored solute is absent, but the solvent and other solutes are the same as in the analyzed solution. By using a blank in this manner the specific effect of the colored solute is isolated from the effects of other factors, which are cancelled by use of the blank. These non-specific factors include about 8 percent loss of light by reflection from the air-glass surfaces of the cuvette, some loss, usually slight, in transmission through the solvent and the glass of the cuvette, and some scattering of light. Although light absorption by a water-clear solvent is usually negligible, sometimes reagents are present which, although not perceptibly colored, significantly lower the transmission. On the other hand, if a cylindrical cuvette is used, its lens effect may so increase the intensity of the transmitted light that the effect more than balances the losses from reflection and nonspecific absorption.

d. The transmission of the colored solute, indicated by T , and measured as $I_a:I_b$ is called the "*transmittance*," or the "*transmittancy*" in order to distinguish it from the total transmission by the cuvette and solution.

151. Use of Beer's Law to Calculate Concentrations. Definitions of Optical Density and Extinction Coefficient

a. To calculate the concentration, c , it is convenient to put equation (1) into logarithmic form by taking the logarithms of both members, and to solve for C :

$$c = \frac{\log T}{l \log t} \quad (2)$$

b. The value, $\frac{1}{\log T}$, or $-\log T$ is the unit of practical calculations. It is called the "optical density" (also the "extinction"), and is designated by the symbol D . The value, $-\log T$, is used in preference to $\log T$ for convenience, because since T is always less than 1 its logarithm is always a negative number; while $-\log T$, or D , is positive, and is proportional to c . For determination of a given colored solute, with constant t and with constant l (cuvettes of constant size), the concentration of colored solute can be calculated as follows:

$$c = k D \quad (3)$$

Equation (3) is derived from equation (2) by substituting D for

$\frac{1}{-\log T}$, and k for $\frac{1}{l \log t}$. The fact that c is in simple direct proportion to D , makes D convenient to use in calculating concentrations.

The physical significance of the optical density is that it is the logarithm of the *attenuation* that the light undergoes as the result of partial absorption by the colored solute. Thus the optical density, $D = 2$, indicates 10², or 100-fold, attenuation of the light, only 1 percent being transmitted, 99 percent absorbed. Similarly, $D = 1$ indicates 10-fold attenuation; $D = 0.6$ indicates 4-fold attenuation; $D = 0$ indicates no attenuation, but complete transmittance, as by a water-clear fluid.*

c. The "*extinction coefficient*," E , is the optical density of solution of unit concentration in a layer 1 cm long. Expressed mathematically, $E = -\log t$. The *molar extinction coefficient*, E_M , is E when the unit of concentration is one mole per liter.

152. Necessity for Monochromatic Light

a. In a colored solution, light of different wave lengths is transmitted in different proportions; this difference is the cause of the color. If the incident light is the white mixture of various wave lengths, and t varies from one wave length to another, neither equation (1) nor the equations derived from it will be valid; optical density will not be proportional to the concentration of colored solute. *The light observed must consist of a band of wave lengths which is so narrow that transmittance is approximately constant over the width of the band.* Otherwise Beer's law does not hold.

b. The tolerated width of the band is different for different colored solutes. If the transmittance factor, t , changes rapidly from wave length to wave length over the visible spectrum, a narrow band is necessary. If, on the other hand, in the curve of transmittance versus wave length, a broad plateau exists where transmittance changes but little, and is low enough to give satisfactory optical densities, a relatively broad band can be used. The statement that photometric analyses can be made only with monochromatic light (light of one wave length) is theoretically exact, but for practical measurement of concentrations of solutions "monochromatic" may be interpreted to mean light within a limited band of the spectrum; how narrow the band must be for a given

* The calculation formula for the Duboscq colorimeter can be derived from Beer's law as follows: If solutions of concentrations c_1 and c_2 are compared, the ratio of the concentrations can be expressed as follows:

$$c_1 : c_2 = \frac{\log T_1}{l_1 \log t} : \frac{\log T_2}{l_2 \log t}$$

The condition for the reading of the Duboscq is that $T_1 = T_2$. Since t is the same for both solutions, the equation simplifies, for the Duboscq reading, to:

$$c_1 : c_2 = l_2 : l_1$$

analysis depends on the solute and the degree of accuracy that is required in the analysis.

153. Choice of Spectral Wave Band or Light Filter for an Analysis

Usually a suitable band is one for which (1) the optical density of the colored solute to be determined is maximal, and (2) which is located, if possible, on a plateau of the curve of optical density versus wave length where the transmittance does not vary much with the wave length. These desirable conditions are often fulfilled when the curve of optical density passes through a maximum in a part of the spectrum that is favorable for observation. However, other factors, such as (3) the optical densities of other solutes that may be present, and (4) the sensitivity of the eye (par. 146) or photoelectric cell to different parts of the spectrum, may also have to be considered. The fact that more than one factor may require consideration makes a choice of the best spectral band or light filter for a given analysis sometimes a complex compromise. Specification of the wave-length band or light filter to be used is accordingly an essential part of the directions for a photometric method of analysis. In table XIII are presented the wave lengths recommended for some of the analyses in general use in clinical chemistry.

154. Types of Photometers

a. Analytical photometers may be classified, according to the devices used to provide light of desired wave length, as spectrophotometers and filter photometers. Spectrophotometers receive the light on prisms or diffraction gratings that disperse it into a spectrum, from which any desired band of waves can be directed into the solution to be analyzed. Such instruments are the Koenig-Martens and the Coleman. Filter photometers isolate light within a desired band of wave lengths by means of colored filters through which the light is passed, either before or after it passes through the analyzed solution. The step-photometer and the Evelyn and Summerson photometers are filter photometers. Filters in great variety may be selected from lists supplied by the Corning Glass Works, Corning, New York, and the Eastman Kodak Company, Rochester 4, New York.

b. Photometers are further differentiated according to the devices by which the transmittances or optical densities are measured.

- (1) In *visual photometers* the intensities of two beams of light are compared visually, one beam passing through the analyzed solution, the other through the blank solution. The beam incident on the blank is diminished by manipulating a calibrated diaphragm (step-photometer) or a set of polarizing prisms (Koenig-Martens) until the two fields match. The fraction to which the light incident on the blank must be diminished to make the two fields match serves as a measure of the optical density of the unknown solution.
- (2) In an *electrophotometer* the intensity of a transmitted light beam is measured by its effect on a photoelectric cell (Summerson, Evelyn, and Coleman photometers). In such a photometer only one beam of light may be needed. The comparison between the blank solution and the unknown can be made by interposing them in succession in the path of the light beam and measuring the relative effects on the photoelectric cell.

155. Photoelectric Cells

Two main types of photoelectric cells are used in photometry. One is the photoemissive cell. In this type electrons are ejected from a metallic surface (inclosed in a tube partly or completely evacuated) when radiant energy strikes the surface. In the other type, commonly called the photovoltaic or barrier cell, the received radiant energy causes a transfer of electrons across a boundary between two dissimilar materials, such as copper and copper oxide, or selenium and a metal, one of these materials serving as a rectifier to keep the transfer of electrons in one direction. Ordinarily the current from one of the modern barrier cells is sufficient to measure directly. The current of a photoemissive cell may require amplification. The design of an electric circuit for an instrument that includes one of these cells requires good engineering. In the directions that go with the instrument the type of cell and spectral range of its sensitivity should be given, as well as its principal features, so that the user can follow the directions intelligently. Of chief importance is a design that makes the electrical output of the cell as nearly proportional as possible to the radiant energy received. The ease of operation of photoelectric instruments often leads the analyst to accept mere pointer readings uncritically. Several instruments use the deflection of the pointer of a galvanometer to indicate some function of the energy absorbed or transmitted by the solution. The meaning of the scale should be understood. In one instrument the scale may indicate

microamperes, in another optical density ($-\log T$), in another there may be an arbitrary scale. Whatever the scale is, it should record results that accord with Beer's law when observations are made on a series of standard solutions of a colored solute that is known to obey that law. Such a solution, which may be used to test the behavior of a photometer is copper sulfate dissolved in 2 *M* ammonium hydroxide. For such copper sulfate solution $E_M = 57.5$ with light of 620 millimicrons wave length, and $E_M = 33.6$ with light of 336 millimicrons.

156. Range of Optical Densities Suitable for Accurate Measurements

In visual photometry it is obvious that if light absorption is too slight, small percentage differences in the absorption will be imperceptible; also that if so much of the light is absorbed that the field is very dark, small differences will again be difficult to estimate. Between these extremes there is a range that is better suited for measurement. For electrophotometers there are also limited practical ranges. It has been estimated (Ringbom) that with ordinary intensities of illumination a visual photometer is most accurate when the optical density observed is from 0.7 to 1.0 (20 to 10 percent transmittance) and that with electrophotometers, as usually constructed for analytical purposes, the desirable range is from optical density of 0.15 to 1.0 (70 to 10 percent transmittance).

157. Photometric Calculations

a. SOLUTIONS THAT FOLLOW BEER'S LAW. If solutions of the colored solute to be determined follow Beer's law within the concentration range covered by the method used, values of c plotted against values of D , observed in analyses of known standard solutions, will fall on a straight line, and the k of equation (3) will be constant. If such is the case, results can either be calculated by formula or estimated by a linear graph.

(1) *Calculation by formula.* The working formula is equation (3), namely, $c = k D$.

- (a) Most convenient for analytical work are photometers equipped with scales from which the optical density, D , may be read directly; on these instruments the blank is set at zero on the scale.
- (b) Other instruments have scales reading in percentage transmittances, usually from 0 to 100; on these the blank is set at 100. When an instrument with a trans-

mittance scale is used, the percentage transmittances are converted into optical densities by the equation:

$$D = 2 - \log \text{ of percentage transmittance.} \quad (4)$$

For example, if the percentage transmittance is 25, its logarithm is 1.398, and the density is $2 - 1.398 = 0.602$. For routine use, with a photometer reading in transmittances, it is convenient to prepare a table with optical densities and the corresponding transmittances covering the useful range of the instrument.

- (c) To make use of equation (3) in determinations of a given colored solute, it is necessary to know the value of k for solutions of this solute in cuvettes of the size used. The value of k is determined by readings on known standard solutions, preferably of two or more different concentrations. Let the known concentration of a standard be c_s , and the reading with it be D_s . Then:

$$k = \frac{c_s}{D_s}. \quad (5)$$

The value of k thus determined is used in equation (3) to calculate c from observed D readings in analyses of unknown solutions.

- (d) If the value of k for a given analytical procedure is at all times constant and reproducible, it is not necessary to reestablish it with each series of analyses; in other words, no standard need be run routinely. It suffices to establish in advance the value of k by careful repeated analyses of standard solutions of different concentrations covering the range to be encountered in analyses of unknown solutions. In establishing the k value, cuvettes must be used giving the same length of transmitting layer of solution that is used in subsequent analyses (the l of equations (1) and (2)), since the validity of equation (3) depends on constancy of l . The possibility of avoiding the necessity of preparing standard solutions with each series of analyses is particularly desirable when the standard solutions are unstable, costly, or difficult to prepare. When a fixed k is used, it is essential in analyses of unknowns that all phases of the analytical procedure, including the wave length or filter used, the quality of reagents and the order of their addition, the temperature of the solution (optical density may change by more than 1 percent per degree of temperature change), the time

of standing before reading, etc., duplicate as closely as possible the conditions prevailing at the time the value of k was established.

(2) *Calculation by comparison of optical density of unknown with optical density of a simultaneously prepared standard.*

- (a) In this procedure a standard is prepared with each series of analyses, as in colorimetric determinations. The calculation is:

$$c = C_s \times \frac{D_u}{D_s} \quad (6)$$

c is the concentration of the unknown, C_s the concentration of the standard, D_u the optical density of the unknown, and D_s the optical density of the standard.

- (b) This procedure is likely to be the one of choice when the intensity of color development, or the reading of the density, depends to such an extent upon conditions that their exact duplication in different series of analyses is difficult. The procedure is recommended in the cholesterol method given in this manual, where the color reaches a maximum and then fades; also in exact determinations of ammonia by nesslerization, where the optical density varies so much with slight variations with wave length that slight differences in adjusting the latter may become a source of variable error if k (equation (5)) is determined at one time, and c (equation (3)) at another time, with another setting of the wave length.

(3) *Graphic calculation.*

- (a) Instead of using equation (3) one may plot c against D on rectangular coordinates, using data obtained from analyses of standard solutions, as described for establishment of k . Because the curve is linear, 2 or 3 points suffice to locate it. By interpolating on the curve the D values obtained in analyses of unknowns, the c values of the latter can be estimated.
- (b) When the photometer has a scale reading in transmittances instead of densities, the graphic estimation is especially convenient, because it avoids the necessity of transposing transmittances, into densities. On semilogarithmic paper the transmittances obtained in analyses of standard solutions are plotted on the logarithmic coordinate and concentrations on the other coordinate. A linear curve is thus obtained on

which concentrations of unknown solutions can be read directly from transmittances.

b. SOLUTIONS THAT DO NOT FOLLOW BEER'S LAW. Not all colored solutions show a linear relation between optical density and concentration. If changes in concentration cause changes in ionization or in other properties affecting transmittance, the curve obtained by plotting c against D will not be linear. Solutions of some substances follow Beer's law in low concentrations but not in higher ones. Apparent failure to follow the law may occur if light of a narrow spectral band is required (par. 153) and the photometer used provides only broader bands, or if the instrument is an electrophotometer that is not so adjusted that the electrical output is proportional to the radiant energy received (par. 155).

If for any reason the equation $k = \frac{c}{D}$ does not give a constant

value for k over the c range of the method, it is necessary to estimate the results of analyses by the graphic procedure outlined in the preceding paragraph. Because the curve is not linear, it is necessary in establishing it to use a number of standards, covering the entire range that may be encountered in analyses, and at sufficiently close concentration intervals to assure the accuracy of all parts of the curve.

Table XIII. Recommended Wave Lengths for Photometric Determinations in This Manual

Determinations	Paragraph No.	Wave length millimicrons
Nitrogen by Kjeldahl digestion and nesslerization -----	191, 192, 193, 194..	450*
Urea by urease hydrolysis and direct nesslerization -----	198, 199 -----	500*
Uric acid -----	200 -----	700
Creatinine -----	201 -----	520
Sugar by Folin-Wu -----	202, 203 -----	520*
Bilirubin by Van den Bergh -----	205 -----	535
Icterus Index -----	206 -----	420
Inorganic phosphorus by Fiske-Subbarow	210, 211 -----	660
Calcium by Roe-Kahn -----	212 -----	660
Sulfonamides by Bratton-Marshall -----	217 -----	540
Sulfonamides by Fuller -----	218 -----	460
Hemoglobin -----	219 -----	545*

* In most instances the wave lengths given are those of maximum optical density. In the instances marked with an asterisk (*), however, authors of the methods have recommended wave lengths other than those of maximal optical density because of special considerations, such as the elimination of errors due to the presence of extraneous colored substances. In such instances it is particularly important to check the linearity of the relation between concentration and optical density with the photometer used.

CHAPTER 4

EXAMINATION OF URINE

Section I. COLLECTION AND PRESERVATION OF SPECIMENS

158. Collection and Preservation

a. SINGLE SAMPLES. A specimen of urine passed at one voiding may be used for qualitative tests.

b. DAY AND NIGHT SAMPLES. To collect these samples, the patient uses two wide-mouthed bottles (each of approximately 1 or 2 liters capacity) — one for all urine passed during the day, and the other for all urine passed during the night. The day period is usually from 6 AM to 6 PM; the night period from 6 PM to 6 AM.

c. TWENTY-FOUR-HOUR SAMPLE. The patient uses a bottle of 2 to 4 liters capacity, as well as a funnel if the bottle is not wide-mouthed. At the beginning of the 24-hour period the first voiding of the morning is discarded, but all urine passed thereafter, including the urine passed on arising on the second morning of the period, is collected. The total volume is recorded, but only 150 to 250 ml of the well mixed sample should be sent to the laboratory, unless more is requested.

d. PRESERVATION.

(1) *Refrigeration.* Clean bottles are essential. Urine without preservatives can be preserved in a refrigerator for about 24 hours. However, freezing must be avoided.

(2) *Chemical preservatives.* Use enough toluene to form a *thin* layer over the surface. It is a good preservative and does not interfere with any chemical tests.

Another effective preservative is formaldehyde, particularly for formed elements. Use no more than 2 drops (0.1 ml) of 40 percent formaldehyde (formalin) per 100 ml of urine, since larger amounts *seriously interfere* with tests for sugar, albumin, and indican.

Section II. PHYSICAL EXAMINATION

159. Physical Properties

a. COLOR. Normal urine varies from almost colorless to a dark orange. The darker shades are usually associated with greater

dissolved solids and higher specific gravities. Hemoglobin and hematin (from blood) are the most important abnormal pigments, giving a dark reddish-blue color in higher or a "smoky" (faint greyish blue-red) color in lower concentrations. In acid urines, these substances give a brown color. Urine containing bile pigment has a dark orange color, and when shaken, the froth is yellow.

b. APPEARANCE (CHARACTER). Freshly voided urine is usually clear. Cloudiness is most frequently due to triple phosphates; more rarely it may indicate some pathological constituent, such as pus. On standing, triple phosphates separate from alkaline urine, and urates from acid urine, causing cloudiness in either case. The phosphates dissolve on addition of acid, urates dissolve when the urine is warmed, but a cloudiness due to pus persists under both conditions.

c. REACTION (APPROXIMATE pH). Place a drop of urine on pH indicator paper ("nitrazine paper" or sodium dinitrophenylazonaphthol disulfonate paper). The pH is determined by matching the color against the colors on the chart that is furnished with the paper. Litmus paper, if available, may also be used; red indicates an acid, blue indicates an alkaline, and an intermediate shade indicates a neutral urine.

d. SPECIFIC GRAVITY. The specific gravity of a sample of urine may be measured by a urinometer, a special hydrometer, which is a weighted glass bulb with a stem carrying a scale of specific gravity. When floating at equilibrium the hydrometer displaces a weight of fluid equal to its own weight. The reading of the scale is made at the meniscus of the air-water interface. The reading in water will be at the 1.00 mark. The reading in urine will be between 1.001 and 1.040. Since increase in the amount of dissolved substances per milliliter increases the specific gravity of urine, a measurement of specific gravity gives an indication of the concentration of total urine constituents.

e. VOLUME. The volume of urine is significant only when the collection interval is known. When this is known the report can be in the form of milliliters per hour, or per 24 hours.

Section III. MICROSCOPIC EXAMINATION OF URINARY SEDIMENTS

160. General

The important formed elements in urine are casts, red blood cells, epithelial cells, leucocytes, and bacteria. Several different

forms of crystals may be seen, but these are rarely of any significance. For appearance of formed elements and crystals, see figures 14 and 15.

161. Casts and Cells

Casts are cylindrically shaped bodies, and are of two kinds. One kind, hyaline casts, are jellylike impressions of kidney tubules. The other kind, cellular casts, are composed of cells sloughed from some part of the genitourinary tract. Hyaline casts dissolve immediately in slightly alkaline water; cellular casts do not. Both kinds of casts may have red blood cells, leucocytes, epithelial cells, or fat droplets adhering to them. Cellular casts may appear granular, or waxy with few or no granules and with little visible cellular structure.

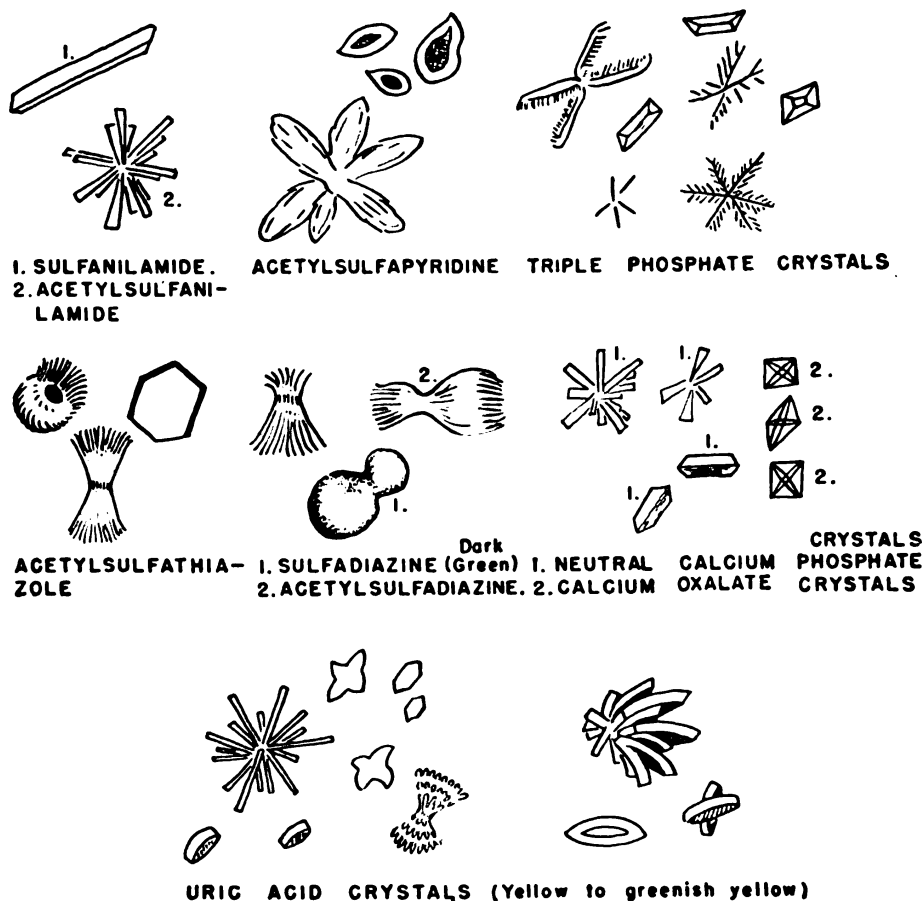


Figure 14. Unorganized elements of urinary sediment. (Except for sulfonamides, urinary crystals are not important.)

162. Qualitative Sediment Examination

Mix and then centrifuge 15 ml of urine, removing most of the supernatant liquid with a pipette. Take up the sediment in a pipette and transfer it to a clean, dry slide or a blood counting chamber. Casts may be viewed under low power, with or without a cover slip, but cells should be examined by high power, with a cover slip over the drop. The best illumination is obtained by lowering the substage condensor and by reducing the iris diaphragm until such objects as red cell "ghosts" and hyaline casts stand out clearly. When a blood-counting chamber is used, the focus should

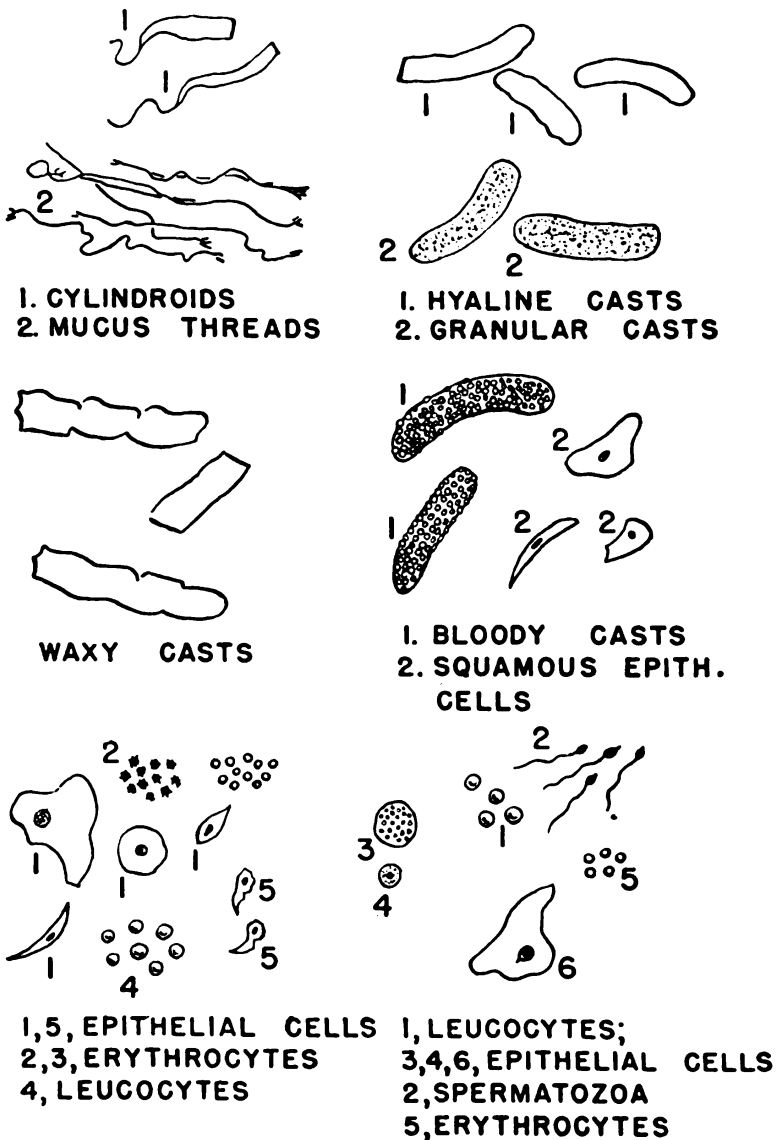


Figure 15. Organized elements of urinary sediment.

be altered slightly during the examination, to define objects that lie at different levels in the chamber. Examine the whole area of the drop for the presence or absence of hyaline casts, cellular casts, red and white blood cells, and other cells. The results of the examination should be recorded as positive or negative for these kinds of casts and cells.

163. Quantitative Sediment Examination

This requires a timed specimen of urine, that is, a collection made between the time when one voiding of urine is discarded and another collected. The volume is measured, and from the time interval over which the urine was collected, the urine volume for $1/5$ of an hour is calculated. For example, let the time be 8 hours, and the volume be 400 ml. Then the required volume is

400
—, or 10 ml. Take the volume for $1/10$ of an hour if that for 8×5

$1/5$ is more than 15 ml. The volume as calculated is placed in a 15-ml graduated centrifuge tube, and spun for a few minutes. With a capillary pipette, remove the supernatant liquid down to the 0.5-ml mark, or to the 0.25-ml mark if the volume for $1/10$ of an hour was taken. The sediment is thoroughly mixed and a drop pipetted to one side of a blood-counting chamber. With a dim light and low power, the casts in two-thirds of the total ruled areas are counted. With high power and a little more light, the red cells, epithelial cells, and white cells in one-fifteenth of the total ruled area are counted. The number of casts observed, multiplied by 100,000 gives the number of casts excreted in 24 hours. The number of cells observed, multiplied by 1,000,000, gives the number excreted in 24 hours.

Section IV. QUALITATIVE CHEMICAL TESTS

164. Routine Tests

In addition to a physical examination (sec. II of this ch.) each urine is usually tested routinely for proteins ("albumin") and for sugar. Other chemical tests are done as requested.

165. Proteins (Albumin)

In testing for albumin, the urine must be absolutely clear. Cloudy samples must be cleared by filtration or centrifuging, lest the presence of small amounts of albumin be obscured.

a. HEAT AND ACETIC ACID TEST.

- (1) *Reagent.* Dilute 1 volume of glacial acetic acid to 10 volumes with distilled water.
- (2) *Procedure.* Fill a test tube two-thirds full of clear urine. Heat the upper portion in a flame until it boils gently. A precipitate or cloudiness in the heated portion may be due to albumin or phosphates. Add 3 to 5 drops of 10 percent acetic acid, 1 drop at a time. If the precipitate dissolves, it is due to phosphates. If it increases, it is due to albumin, but *only* if the urine was clear before heating. Positive albumin tests are classified as follows: faint turbidity, +; heavy turbidity, ++; precipitate heavy but not solid, +++; boiled solid, ++++. If Bence-Jones protein is present, a turbidity will appear upon heating the urine to 60° C., the turbidity decreasing at higher temperatures and reappearing when the tube is cooled.

b. SULFOSALICYLIC ACID TEST.

- (1) *Reagent.* Dissolve 20 gm of sulfosalicylic acid in water and make up to 100 ml.
- (2) *Procedure.* To 2 ml of clear urine (filtered if necessary), add 6 drops of the sulfosalicylic acid reagent. Do not heat. Turbidity or cloudiness indicates protein. The same classification is used as in the acetic acid test, except that for ++++, solid coagulation does not occur.

166. Sugar

a. BENEDICT'S QUALITATIVE REAGENT. Dissolve 17.3 gm of cupric sulfate crystals ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$) in about 100 ml of water. Dissolve 117 gm of monohydrated sodium carbonate ($\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$), (or 100 gm of anhydrous sodium carbonate), and 173 gm of sodium citrate in about 700 ml of water by warming. Cool to room temperature. Pour the copper sulfate solution into the carbonate-citrate solution slowly with constant stirring. When the solutions are completely mixed, dilute with water to a volume of 1 liter and mix again.

b. PROCEDURE. To 5 ml of reagent in a test tube, add 8 drops (0.5 ml), but no more, of urine. Heat for 5 minutes in an actively boiling water bath. Cool in air. If no sugar is present, the solution will remain clear blue, or show a slight greenish-blue turbidity due to urates. But if 0.1 gm or more of glucose per 100 ml is present, a bulky precipitate appears; green (trace), greenish-yellow (+), yellow (++), orange (+++), or brick red (++++), as the amount of glucose increases.

167. Acetone

a. SODIUM NITROFERRICYANIDE (NITROPRUSSIDE) TEST (LANGE'S).

(1) *Reagents.*

- (a) Acetic acid, glacial, 99 percent.
- (b) Ammonium hydroxide, concentrated 28 percent, strong ammonia water.
- (c) Sodium nitroferricyanide, freshly prepared saturated solution. Dissolve several crystals in 1 to 2 ml of water by gentle heat. Have a slight excess of undissolved crystals remaining.

- (2) *Procedure.* Place 5 ml of filtered urine in a test tube, add 0.5 ml of glacial acetic acid and 0.5 ml of the freshly prepared sodium nitroferricyanide solution, and mix. Tilt the tube and carefully overlay the mixture with 1 to 2 ml of strong ammonia water.

- (3) *Results.* A purple or purplish-red ring forms at the contact zone in a few minutes if acetone is present. The ring tends to be more purple or violet in low concentrations, more red-purple in high. Amorphous urates may give a brown or orange ring if present in large amount. This test detects the presence of both acetone and acetoacetic acid. The test is, however, much more sensitive for acetoacetic acid. Acetoacetic acid gradually decomposes into acetone and carbon dioxide, particularly if the urine is acid and stands in a warm room. Because of the difference in sensitivity of the test for the two compounds, the intensity of color obtained may decrease if urine is permitted to stand before it is tested. Hence the test should be done with fresh urine.

b. SALICYLIC ALDEHYDE TEST (BEHRE).

(1) *Reagents.*

- (a) Fifty percent sulfuric acid (concentrated sulfuric acid added to an equal volume of distilled water).
- (b) Salicylic aldehyde.
- (c) Thirty-two percent sodium hydroxide solution.

- (2) *Procedure.* Place 3 ml of urine in a clean, dry test tube and add 1 drop of 50 percent sulfuric acid. Prepare a thin square of cotton of somewhat greater width than the test tube and drop on the center of the square 1 drop of salicylic aldehyde, and over this 2 drops of 32 percent NaOH solution. The two reagents produce a solid in the

form of a flat yellow disk on the cotton. Invert the cotton over the mouth of the test tube so that the disk formed by the reagents is turned down toward the urine. Push the cotton down slightly into the tube so that it is held in place and so that the disk of reagents does not touch the side of the tube. Place the tube upright in a boiling water bath for 8 minutes. Then remove the cotton and note change in color of the reagent disk.

- (3) *Results.* In the presence of as little as 1 mg of acetone per 100 ml of urine, or its equivalent in acetoacetic acid (which is changed to acetone by heating with acid) a distinct pink color develops on the disk. With normal urines the yellow color of the disk remains unchanged.

168. Bile in Urine

a. **COLOR.** Shake vigorously a few ml of urine in a test tube. If bile is present, the foam will have a yellow color when compared to the foam of urine without bile.

b. **BILIRUBIN (URINE).** The *Watson modification of the Harrison Test* is one of the most sensitive tests for urine bilirubin and is indicated in all patients suspected of having liver disease. It is important to realize that bilirubin may be detected in the urine when the concentration of total serum bilirubin is still within the normal range. The modification of Harrison's Test as proposed by Watson is carried out as follows: Immerse a strip of barium chloride-impregnated* filter paper into the urine sample for a depth of about $\frac{3}{4}$ inch and after approximately 1 minute remove and allow the excess urine to drain off by touching the strip to the top of the container. Lay the strip upon any absorbent paper, for example, paper towel, newspaper, etc. Now add one or two drops of Fouchet's† reagent at the upper zone or line marking the depth to which the strip of paper has been immersed, not to be confused with the spread of moisture due to capillary action above this point. In the presence of bilirubin a green or greenish-blue color will develop. The color is produced by the reaction of ferric chloride with the barium compound resulting from its combination with bilirubin. If a large amount of bilirubin is present, a deep green color of wide extent will be seen; if a small amount, a faint green line or band will appear across the strip.

* Soft, thick absorbent paper, such as Schleicher & Schuell #470, dipped in a saturated aqueous solution of barium chloride dried and cut into strips approximately 4 inches by $\frac{1}{2}$ inch.

† Fouchet's reagent is a mixture of 25 gm trichloroacetic acid, 0.9 gm ferric chloride and 100 ml distilled water.

169. Urobilinogen

a. REAGENT. Dissolve 20 gm of p-dimethylaminobenzaldehyde in 150 ml of concentrated hydrochloric acid (sp. gr. 1.19), and add 150 ml of water.

b. PROCEDURE. Add 1 ml of reagent to 10 ml of fresh urine. A cherry-red color indicates the presence of urobilinogen. If delay is necessary, keep the urine cold and protected from light but run the test as soon as possible since urobilinogen tends to change into urobilin, which does not react.

Section V. QUANTITATIVE CHEMICAL ANALYSES

170. Protein (Albumin), Sedimentation Method of Shevky and Stafford¹

a. REAGENTS. Tsuchiya's solution: Mix 15 gm of phosphotungstic acid, 50 ml of concentrated hydrochloric acid, and 1,000 ml of 95 percent ethyl alcohol.

b. PROCEDURE. Nephritic urines are usually diluted tenfold. In urines with very scanty protein content a lesser dilution or none at all will give more exact results. Occasionally a urine is encountered with more than 2.8 percent of protein, which is the maximum that can be determined with a tenfold dilution. In such a case a fresh sample is diluted twentyfold and the determination repeated. Of the diluted urine, 4 ml are measured into a special graduated centrifuge tube (Shevky-Stafford tube), the 4-ml mark on the tube itself serving for the measurement. Tsuchiya's reagent is added to the 6.5-ml mark. Mix the contents well by inverting the tube several times, allow to stand exactly 10 minutes, and centrifugalize for exactly 10 minutes at 1,800 rpm. The volume of precipitate is read on the scale in hundredths of a milliliter.

c. CALCULATION. *Grams of protein per liter of urine* = *ml of precipitate* $\times$ 7.2 $\times$ *dilution*. "Dilution" indicates the number of times the urine was diluted before the sample was measured into the tube.

171. Sugar (Benedict)²

a. REAGENTS.

(1) Pumice or talc.

(2) Sodium carbonate.

(3) Benedict's quantitative reagent. Dissolve 87.0 gm of sodium carbonate. ($\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$) (or 74 gm of anhyd-

¹ Archives of Internal Medicine, 32,222 (1923).

² Journal of American Medical Association, 57,1193 (1911).

rous Na_2CO_3), 200 gm of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and 125.0 gm of potassium thiocyanate (KCNS) by warming in enough water to make about 800 ml of solution. Filter if necessary. Dissolve exactly 18.00 gm of copper sulfate ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$) separately in about 100 ml of water, and pour this solution slowly with constant stirring into the carbonate solution. Only the copper sulfate need be weighed exactly. While the solution is still warm, add 0.25 gm of potassium ferrocyanide (yellow prussiate) dissolved in 5 ml or more of distilled water. Cool the solution to room temperature. Transfer the solution to a 1-liter volumetric flask, rinse the container, used in preparing the solution, thoroughly into the volumetric flask with distilled water, make to exactly 1 liter, and mix. Exactly 25 ml of this reagent should be equivalent to 50 mg of glucose. The solution is tested by titration against a standard 2 percent glucose solution and adjusted, if necessary, by diluting or by adding copper sulfate.

b. PROCEDURE.

- (1) Before the titration the urine is diluted, if necessary, so that it contains not more than 1 percent of sugar. Urine from uncontrolled diabetes, with ++++ qualitative sugar reaction ordinarily is diluted 10 times. Other urine is not diluted. Fill a 50 ml burette with the diluted or undiluted urine. Pipette exactly 25 ml of reagent into a 250 ml (115-mm diameter) porcelain evaporating dish. Add a small amount of pumice (preferably) or talc to promote smooth boiling. Add 7.5 gm of sodium carbonate ($\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$) or 6.0 gm of anhydrous sodium carbonate (Na_2CO_3). Heat to boiling and keep mixture boiling vigorously during the titration. Stir with a glass rod if an evaporating dish is used, or by rotation if a flask is used. When the carbonate has completely dissolved, add the diluted urine from the burette at the rate of about 2 drops per second until a chalk-white precipitate forms and the blue color begins to fade. The diluted urine should not be added so rapidly that the temperature is lowered below the boiling point. At the end of the titration allow $\frac{1}{2}$ -minute intervals between additions of urine, which are made 3 or 4 drops at a time. Blue crusts around the edges of the boiling mixture must be pushed into the boiling liquid with the glass rod. If much crust forms at edges of the boiling mixture, add a little more

water. The end-point is the disappearance of the last trace of blue from the hot liquid.

- (2) The volume of liquid must be kept constant at about 25 ml by boiling off water at the same rate at which the urine is added. If the volume is permitted to increase much, the results are not accurate. The end-point must be determined while the solution is still hot since, if the solution is allowed to cool the reaction tends to undergo reversal and the solution will reassume a bluish-green tint. With pure glucose the final mixture is entirely colorless except for the grayish appearance imparted by the pumice. With urine, a slight yellowish-green color due to urinary pigments remains even after the copper has been entirely reduced.

$5d$

c. CALCULATION. Grams glucose per 100 ml urine = $\frac{5d}{C}$. d is

the number of times the urine is diluted. C is the number of ml of the diluted urine used in the titration. When d is 10 the calculation simplifies to:

$$\text{Grams glucose per 100 ml urine} = \frac{50}{C}$$

172. Urea and Ammonia (Urease-aeration method (Van Slyke and Cullen,³ modified by Van Slyke and Hiller⁴) for titration in boric acid solution)

a. GENERAL.

- (1) Urea is hydrolyzed by the enzyme, urease, into ammonium carbonate: $\text{CO}(\text{NH}_2)_2 + 2 \text{H}_2\text{O} \rightarrow (\text{NH}_4)_2\text{CO}_3$. Phosphate buffer is added to give a pH of 6.5 to 7.5 for optimum activity of the enzyme during the reaction: without the buffer the ammonium carbonate would make the solution too alkaline for the best action of the enzyme. From the ammonium carbonate the NH_3 is set free by addition of saturated K_2CO_3 solution, and is driven by a rapid current of air into a 4-percent solution of boric acid. (The 4-percent boric acid solution is sufficiently acid to prevent the loss of any significant amount of ammonia in the air current, but a 2-percent boric acid solution is not sufficient to prevent measurable loss.) The ammonia caught in the boric acid is measured by titrat-

³ Journal of Biological Chemistry, 19,211 (1914) and 24,117 (1916).

⁴ Unpublished.

ing with H_2SO_4 back to the original pH of the acid, as described in paragraph 123e and f.

- (2) The ammonia measured by this procedure includes the nitrogen of both the urea and the pre-formed ammonia of the urine. The pre-formed ammonia is determined in a separate analysis in which the urease is omitted. Subtracting the pre-formed NH_3 nitrogen from the NH_3 + urea nitrogen gives the urea nitrogen.

b. REAGENTS.

- (1) *Urease, 10 percent solution.* "Double strength" urease is dissolved in 10 ml of water for each gram of the urease. The urease powder is weighed into a small flask or cylinder and a small part of the water is stirred in, enough to make a paste. Then the rest of the water is added in portions. If kept in a refrigerator the urease solution will retain its activity and form no NH_3 for several days, but it is preferable as a routine to prepare only enough solution for each day's analyses.
- (2) *Phosphate buffer.* Six gm of KH_2PO_4 and 2 gm of anhydrous Na_2HPO_4 (or 5 gm of crystalline $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) are dissolved in water and diluted to 1 liter.
- (3) *Saturated potassium carbonate solution.* Nine hundred gm of dry granular K_2CO_3 are dissolved in 1 liter of water.
- (4) *Sulfuric acid, 0.01071 N solution.* Fill a 100-ml volumetric flask to the mark with 0.1 N sulfuric acid. Transfer the solution with three washings to a 1-liter volumetric flask. Add to the 1-liter flask from a burette 7.10 ml more of the 0.1 N acid. Fill to the 1-liter mark and mix.
- (5) *Boric acid, 4-percent solution.*
- (6) *Bromcresol green indicator.* A 0.1 percent solution in alcohol. Dissolve 100 mg. of indicator in 100 ml of 95 percent alcohol. Place in dropping bottle and mark the bottle with the number of drops required to deliver 0.1 ml.
- (7) *Caprylic alcohol.*

c. APPARATUS.

- (1) The apparatus is shown in figure 16. In addition are required large test tube racks, each rack holding eight tubes. The tubes are of heavy-walled Pyrex glass, 32 by 200 mm. The lower ends of the inlet tubes end in small bulbs (Folin) with pinhole perforations to break the air into small bubbles. The rubber connecting tubes are about 6 inches long, and are of thick-walled stethoscope

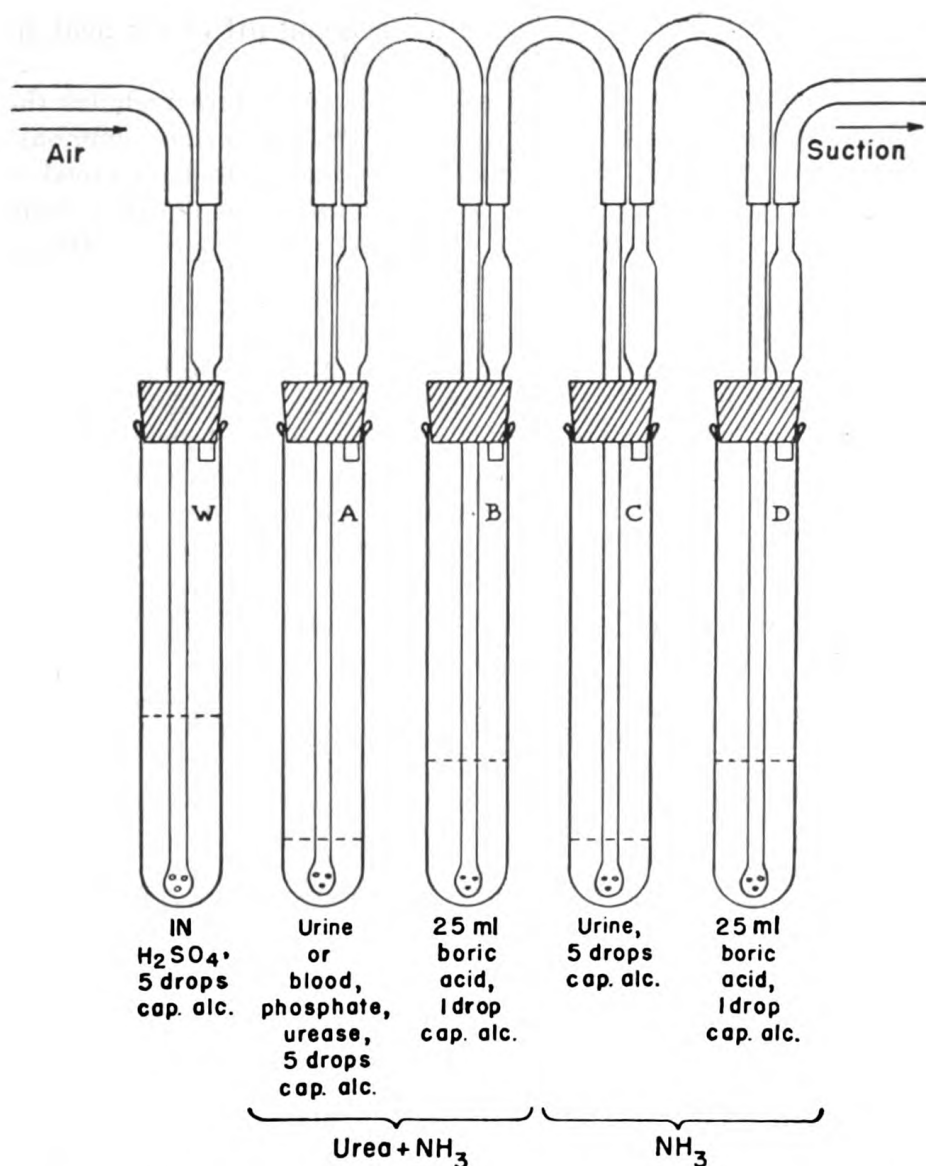


Figure 16. Aeration apparatus for urea and ammonia determinations.

tubing. These rubber tubes must be specially cleaned and freed of sulfur or they may absorb measurable amounts of ammonia. They are scrubbed out with burette brushes, then boiled for 30 minutes in 10 percent NaOH, making sure that the bores of the tubes are filled with the solution. The tubes are then washed, first in tap water, then in distilled water. They are never washed with acid.

- (2) Fifteen or twenty pairs of test tubes can be connected in series and aerated with suction from a single pump. A

laboratory with many routine blood and urine urea analyses may use 50 or more of these tubes. Tube *W* serves as a wash bottle to remove ammonia from atmospheric air. One such tube serves for as long a train of A-B-C-D groups as are attached to one pump.

d. PROCEDURE.

(1) *Setting up apparatus and digesting with urease.*

- (a) Into each of tubes B and D (fig. 16) measure 25 ml (± 0.1 ml) of the 4-percent boric acid, 0.7 ml of brom-cresol green solution, and 1 drop of caprylic alcohol. Set the stoppers in place, without the rubber connecting tubes.
- (b) Dilute 5 ml of urine to 50 ml. Into tube A measure 3 ml of tenfold diluted urine, 3 ml of the phosphate buffer, 2 drops of caprylic alcohol, and 0.5 ml of the urease solution. As soon as the urease is added close the tube with the stopper, without attaching the rubber connecting tubes, and mix.
- (c) While the enzyme is acting in tube A measure 3 ml of the undiluted urine into tube C for NH_3 determination, and add 2 drops of caprylic alcohol.
- (d) To the wash bottle (*W*, fig. 16) add 5 drops of caprylic alcohol.

(2) *Aeration.*

- (a) After the urease has acted for the required time found in the activity test, connect tube C with D, and D with suction, and start a *slow* current of air. While the current is running add to C 10 ml of the saturated K_2CO_3 solution by inserting the tip of a 10 ml graduated pipette, with a wide outlet, into the inlet tube of C. Then connect C with B and B with A, and similarly admit 10 ml of K_2CO_3 solution into A. Then at once connect A with the wash bottle *W*, so that the series is arranged as shown in figure 16. Run the air current slowly for 2 minutes more, then speed it up to its regular rate, which may be as fast as 5 liters per minute. The air current can be run at the full speed obtainable with an ordinary water pump. The most likely source of error is running the air current too slowly.
- (b) If several analyses are to be done, other groups of four tubes each can be inserted in the train between D and the suction pump, and all can be treated and aerated together.

- (c) The aeration is continued for the length of time found necessary for quantitative transfer of NH_3 with the air current used (see below).
- (d) The tubes are then disconnected, *beginning with the end at which the wash bottle is attached*. In a long series of tubes those nearest the pump are under considerable negative pressure, and if the connection were first broken by disconnecting next to the pump, liquid in some of the tubes at that end of the series might be drawn back into the preceding tubes.
- (3) *Titration*. The ammonia in the boric acid tubes is titrated with 0.01071 *N* sulfuric acid delivered from a 25-ml burette. A control tube for the end-point is prepared by adding 25 ml of 4-percent boric acid, 25 ml of water, and 2 drops of bromcresol green solution to a tube of the same size and shape as those in the series. The 0.01071 *N* sulfuric acid is added to the titrated tube until the blue color changes to approach the green of the control. Then water is added to the titrated tube to bring its volume nearly up to that of the control, and addition of 0.01071 normal sulfuric acid is continued until the color matches that of the control.
- (4) *Blank analysis of reagents*. Blank analyses are performed on the reagents added to tube A and to tube C. The potassium carbonate especially is likely to contain traces of ammonia.
- (5) *Calculation*. $\text{Mg urea N} + \text{ammonia N per 100 ml urine} = 50 (A_1 - C_1)$. A_1 is the number of ml of 0.01071 *N* sulfuric acid required to titrate the ammonia caught in tube B. C_1 is the number of ml of 0.01071 *N* sulfuric acid required to titrate the ammonia caught in tube B in a blank analysis on the reagents in tube A.
- Mg ammonia N per 100 ml of urine* $= 5 (A_2 - C_2)$. A_2 is the number of ml of 0.01071 *N* sulfuric acid required to titrate the ammonia in tube D. C_2 is the number ml of 0.01071 *N* sulfuric acid required to titrate the ammonia caught in tube B in a blank analysis of the reagents in tube C.

$$\text{Urea N} = (\text{urea N} + \text{ammonia N}) - (\text{ammonia N}).$$

e. POINTS TO BE NOTED IN DETERMINATION OF AMMONIA BY AERATION.

- (1) The time required for complete transfer of ammonia by aeration is ascertained as follows: Place 5 ml of a 0.6 percent ammonium sulfate solution, 10 ml of potassium carbonate solution and 2 drops of caprylic alcohol in tube

A of figure 16, and connect with tube B containing 25 ml of 4 percent boric acid, 2 drops of bromocresol green indicator solution and 1 drop of caprylic alcohol. Pass a current of air at the same speed as that used in urea analyses. At 5-minute intervals replace the boric acid receiving tube with successive fresh tubes of boric acid. Compare the color of the indicator in the boric acid with that in a control tube of 4 percent boric acid and indicator. When a boric acid tube from the train after 5 minutes' aeration shows the same color as the control tube, indicating that no more ammonia is being evolved, the aeration is completed. With a good water pump aeration can be completed in 15 or 20 minutes.

- (2) A slow current of air should be used *during the first 2 minutes after the potassium carbonate is added*. Otherwise ammonia may be driven over so rapidly at the start that a slight amount escapes absorption by the boric acid. After the first 2 minutes one may use a current of 5 liters per minute. In order to transfer all the ammonia in the apparatus set up exactly as described, 75 liters of air is sufficient (25 liters transfers 98 percent). The length of time required to complete the aeration depends on the interval required to draw through the necessary volume of air.
- (3) The inlet tubes in test tubes A and C, figure 16, must reach to the bottoms of the test tubes.
- (4) The potassium carbonate is used for two effects: (1) It liberates the ammonia by making the solution alkaline, (2) the concentrated potassium carbonate diminishes greatly the solubility of ammonia in the water, and thereby shortens the time required for the aeration. Potassium carbonate cannot be replaced by sodium carbonate, because the latter does not have as great an accelerating effect on the evolution of NH_3 .
- (5) The time required for the urease to decompose the maximal amount of urea that may be present in urine is ascertained by setting up in series four pairs of tubes, as directed for tubes A and B, figure 16, with 0.3 percent urea solution in place of tenfold diluted urine, and adding the potassium carbonate to the urea-containing tubes after the urease has acted for 5, 10, 15, and 30 minutes, respectively. The train of tubes is then aerated and the ammonia in the boric acid tubes is titrated. From the results the length of time is ascertained that the urease must act to give the full yield of ammonia. The time

interval thus ascertained is valid for the temperature at which the test is made. A rise of 10° C. in temperature shortens the time to half, and a 10° fall in temperature doubles the necessary time interval, for the urease to act.

- (6) *Separate sets of tubes and stoppers* must be employed as containers for the *boric acid*, and for the *urine-potassium carbonate solutions* respectively, because after concentrated alkali carbonate has been in the tubes it is difficult to wash off with water all traces of the alkali. Enough may remain to affect the titration if the same tube is used for boric acid. Furthermore, if a tube with boric acid adherent in it is used for the urease digestion, the urease may be inactivated by the boric acid. The tubes and stoppers used for boric acid are marked, and not used for other purposes.
- (7) Tubes which are used for Nesslerization cannot safely be employed for urea decomposition by urease. The adherent trace of mercury salt may inactivate the urease.

173. Urea

(Colorimetric (Gentzkow and Masen)⁵)

a. PRINCIPLE. The urea is hydrolized to ammonium carbonate by urease as in the foregoing method, but the ammonia is determined by clearing the solution with tungstic acid and Nesslerizing the filtrate. The procedure is somewhat less exact than the aeration-titration method, and colorimetry has the disadvantage that great variations in the urea concentration of urine lead to colorimetric comparisons in which standards and unknown differ outside the desirable 1:2 to 2:1 limits. This disadvantage can be overcome by repeating such analyses with larger or smaller samples of urine.

b. REAGENTS.

- (1) The reagents listed for colorimetric blood urea (par. 198).
- (2) Permutit powder.
- (3) Acetate buffer. Dissolve 15 gm of crystalline sodium acetate, ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3 \text{H}_2\text{O}$) in 50 to 75 ml of water in a 100-ml volumetric flask, add 1 ml of glacial acetic acid, and dilute to 100 ml with water.

c. PROCEDURE.

- (1) *Preparation and digestion of urine.* To 5 ml of urine in a 25-ml Erlenmeyer flask add 2 gm of permutit and shake for 5 minutes with a rotary motion to absorb the pre-

⁵ Journal of Biological Chemistry. 143,531 (1944).

formed ammonia from the urine. Filter. Dilute 2 ml of the NH_3 -free filtrate to 100 ml. Pipette 5 ml of the diluted urine into a 50-ml volumetric flask. Add 25 to 30 ml of water, 1 ml of acetate buffer solution, and 20 mg of "double strength" urease powder, or 0.2 ml of a 10 percent urease solution. Mix and let stand at room temperature the length of time necessary to give a 100 percent yield of ammonia from urea in control analyses with 3 percent urea solutions. (Twenty minutes should suffice.) Then add 2.5 ml of the 10 percent sodium tungstate, 2.6 ml of 0.67 *N* sulfuric acid, and water to bring the volume to the 50-ml mark. Mix and filter through a dry, ammonia-free filter of good "qualitative" grade, refiltering if necessary to obtain an absolutely clear filtrate. The urine has now been diluted 500-fold, and the concentration of ammonia nitrogen formed from the urea will ordinarily approximate the urea nitrogen concentration of a 1:10 blood filtrate.

(2) *Nesslerization and color measurement.*

(a) A 5-ml portion of urine filtrate is Nesslerized and the color compared with that developed in a standard solution containing 0.015 mg of ammonia nitrogen per ml, as described in paragraph 198, for determination of the ammonia in filtrate of urease-treated blood. The Nesslerized filtrate is compared with the standard in either a colorimeter or a photometer, as described in paragraph 198*d*(4).

(b) If in the colorimetric or photometric reading the color proves to be too weak or too deep for accurate measurement, the Nesslerization is repeated, with a portion of urine filtrate greater or less than the 5 ml first used.

d. CALCULATION.

(1) *Colorimetric.* When a 5-ml portion of filtrate, representing 0.01 ml of urine, is Nesslerized and compared with a standard of 0.075 mg of ammonia nitrogen, the calculation is:

$$\text{Mg urea nitrogen per 100 ml urine} = 750 \frac{S}{U}$$

where *S* is the reading of the standard and *U* the reading of the unknown. If a volume of filtrate other than 5 ml is used, the calculation is:

$$\text{Mg urea nitrogen per 100 ml urine} = \frac{3750}{V} \times \frac{S}{U}$$

where *V* is the ml of filtrate taken.

- (2) *Photometric.* When 5 ml of urine filtrate are Nesslerized the Nesslerized solution represents a 2,500-fold dilution of the urine. The calculation is therefore:

$$\text{Mg urea nitrogen per 100 ml urine} = 750 \times \frac{D_u}{D_s}$$

D_u is the optical density of the Nesslerized filtrate, D_s the density of the standard.

If a volume of urine filtrate, V , other than 5 ml is taken, the calculation is:

$$\text{Mg urea nitrogen per 100 ml urine} = \frac{3750}{V} \times \frac{D_u}{D_s}$$

174. Chloride

a. The procedure for chlorides in urines of ordinary chloride content (above 50 millimoles per liter) is the same described for plasma (par. 209), 1 ml of urine being mixed with 25 ml of phosphoric-tungstic acid solution and shaken with silver iodate, after which 10 ml of the filtrate is titrated with 0.2038 normal thiosulfate.

b. When the urine chloride is so exceptionally low that less than 5 ml of thiosulfate solution is used in the titration, repeat the analysis, taking a sample of 5 ml of urine, instead of 1 ml. The 5-ml sample is mixed with 25 ml of the phosphoric-tungstic acid solution, and the rest of the analysis performed as usual. With urines so low in chloride, the larger sample is required for a precise result.

c. CALCULATION WHEN URINE SAMPLE IS 1 ml.

Millimoles chloride per liter urine = $10 \times \text{ml thiosulfate}$.

Grams NaCl per liter urine = $0.585 \times \text{ml thiosulfate}$.

d. CALCULATION WHEN URINE SAMPLE IS 5 ml.

Millimoles chloride per liter urine = $2.31 \times \text{ml thiosulfate}$.

Grams NaCl per liter urine = $0.135 \times \text{ml thiosulfate}$.

175. pH of Urine

a. REAGENT.

- (1) *Standard buffer solutions.* The phthalate and phosphate solutions described in section V of chapter 3.
- (2) *Indicator solutions.* 0.04 percent solutions of brom cresol green, brom cresol purple, and phenol red. (See par. 123.)

b. **COLLECTION OF URINE.** If urine is exposed to air, loss of CO_2 may cause a rise in pH. Consequently the urine is collected with least possible exposure to air and a portion for the pH determination is placed at once in a bottle, which is filled so that only a small air bubble is left, and is stoppered. The pH measurement is carried out as soon as possible, because on standing bacterial formation of ammonia may quickly cause a gross increase in pH.

c. **PH MEASUREMENT.** In each of three test tubes place 5 ml of water. Into the three tubes measure respectively, 0.4 ml brom cresol green, 0.2 ml of brom cresol purple, and 0.2 ml of phenol red. To each tube add 1 ml of urine from a 5-ml graduated pipette which dips below the surface during the delivery. Dilute to 10 ml with water and mix the contents of each tube by stirring with a footed rod. From the colors judge which indicator is suitable for the measurement and compare the selected tube with buffer standards as described in paragraph 122.

176. Sulfonamides

a. **GENERAL.** The reactions and conditions of the Bratton-Marshall method and the Fuller method are discussed, respectively, in paragraphs 217 and 218, in which the methods are applied to blood analyses. Here will be given only the differences in preparation of material and in calculation required for urine analyses. *Reagents and standard solutions* are the same described for blood in paragraphs 217 and 218.

b. BRATTON AND MARSHALL'S METHOD.

(1) *Preparation of urine.*

(a) *Preparation for colorimetric measurement.* The urine is diluted 500-fold in two stages. First 1 ml of urine is diluted with distilled water to 50 ml (first dilution). Then 5 ml of the fiftyfold diluted urine is pipetted into another 50-ml flask, and about 20 ml of water is added. If the urine is protein-free, 2 ml of the 4 N HCl is then added; if the urine contains protein, 10 ml of the 15 percent trichloroacetic acid is added in place of the HCl. After addition of either acid, the volume is made up to 50 ml, and the liquids are mixed (second dilution). If protein is present, the precipitate formed with trichloroacetic acid is filtered off.

(b) *Preparation for photometric measurement.* The urine is diluted 1,000-fold in two stages. For the first dilution 1 ml of urine is diluted to 100 ml. The second dilution is 5 ml to 50, with addition of HCl or trichloroacetic acid, as for preparation for colorimetric measurement.

- (2) *Development of color to measure free sulfonamide.* This is carried out, in the manner described for blood filtrate, on a 10-ml portion of either the 500-fold or the 1,000-fold diluted urine, according to whether the measurement is to be colorimetric or photometric.
- (3) *Development of color to measure total sulfonamide.* The hydrolysis with HCl is carried out on a 10-ml portion of the diluted urine as described for total sulfonamide in blood filtrates. In the diluted protein-free urine, however, since HCl is added in the final dilution no more need be added before heating to hydrolyze conjugated sulfonamide. If trichloroacetic, instead of hydrochloric, acid is added in the "second dilution," 0.5 ml of the 4 N HCl is added, as in blood filtrates, before heating.
- (4) *Development of color in standards.* As for blood analyses.
- (5) *Colorimetric measurement.* As for blood analyses. However, because of the variation in the concentration of sulfonamides in urine, some samples when diluted 500-fold may give too much or too little color for accurate measurement. In such a case another "second dilution" is prepared. Of the fiftyfold dilute urine, prepared by the "first dilution," pipette into a 50-ml flask a volume greater or less than the usual 5 ml, according to whether a stronger or a weaker color is needed. Then add about 20 ml of water, followed by HCl or trichloroacetic acid, made up to 50 ml, as directed under (1) above, develop the color in 10 ml, and repeat the colorimetric measurement.
- (6) *Colorimetric calculation.* When the color is developed in the 500-fold diluted urine the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = 500 \times C_s \times \frac{S}{U}$$

C_s is the mg of sulfonamide per 100 ml of the working standard solution, S is the reading of the standard, U the reading of the unknown.

When the dilution is other than 500-fold, the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = \frac{2,500}{V} \times C_s \times \frac{S}{U}$$

V is the ml of the fifty-fold diluted urine that is diluted to 50 ml in the "second dilution."

- (7) *Photometric measurement.* This is done as described for blood analyses.

If the 1,000-fold dilute urine gives too little or too great color intensity, repeat the "second dilution," as described in (5) above, diluting a proper volume of the hundredfold diluted urine to 50 ml, with addition of HCl or trichloroacetic acid, and repeat the photometric measurement.

- (8) *Photometric calculation.* When the color is developed in the 1,000-fold diluted urine the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = 200 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown, D_s the density of the standard with 0.2 mg of sulfonamide per 100 ml.

When the dilution is other than 1,000-fold, the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = \frac{1,000}{V} \times \frac{D_u}{D_s}$$

V is the ml of hundredfold diluted urine diluted to 50 ml in the "second dilution."

c. FULLER'S METHOD.

- (1) *Preparation of urine.*

(a) *Preparation for colorimetric measurement.* The urine is diluted 250-fold in two stages. First 2 ml of urine is diluted with distilled water to 50 ml (first dilution). Then 5 ml of the twenty-five-fold diluted urine is pipetted into another 50-ml flask. Ten ml of the 5 percent trichloroacetic acid is added, followed by water to make the volume 50 ml (second dilution). If a precipitate of proteins appears, filter.

(b) *Preparation for photometric measurement.* The urine is diluted 500-fold in two stages. The first dilution is 1 ml of urine to 50. The second dilution is 5 ml of the first to 50, with addition of trichloroacetic acid, as described above.

- (2) *Development of color to measure free sulfonamide.* This is carried out, in the manner described for blood filtrate, on a 10-ml portion of the 250- or the 500-fold diluted urine, according to whether the measurement is to colorimetric or photometric. However, when the alkaline thymol solution is added to urine a precipitate of calcium and magnesium salts usually forms. This is filtered off and the clear filtrate is used for measurement of the color.

- (3) *Development of color to measure total sulfonamide.* The hydrolysis with HCl and the color development are as described for total sulfonamide in blood filtrate. Filter off calcium and magnesium salts that precipitate after addition of alkaline thymol solution.
- (4) *Development of color in standards.* As for blood analyses.
- (5) *Colorimetric measurement.* As for blood analyses. However, because of the variation in the concentrations of sulfonamides in urine, some samples when diluted 250-fold may give too much or too little color for accurate measurement. In such a case another "second dilution" is prepared. Of the twenty-five-fold diluted urine prepared by the "first dilution," pipette into a 50-ml flask a volume greater or less than the usual 5 ml, according to whether a stronger or weaker color is needed. Then add 10 ml of 5 percent trichloroacetic and complete the "second dilution" to 50 ml. Repeat the colorimetric measurement with 10 ml of this dilution.
- (6) *Colorimetric calculation.* When the color is developed in the 250-fold diluted urine the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = 250 \times C_s \times \frac{S}{U}$$

When the dilution is other than 250-fold, the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = \frac{1,250}{V} \times C_s \times \frac{S}{U}$$

V is the ml of the twenty-five-fold diluted urine that is diluted to 50 ml in the "second dilution." C_s is the mg sulfonamide per 100 ml of working standard.

- (7) *Photometric measurement.* This is as described for blood analyses. If the 500-fold diluted urine gives too little or too great color intensity, repeat the "second dilution," as described above for colorimetric measurement in urine, diluting a proper volume of the fifty-fold diluted urine to 50 ml with trichloroacetic acid and water. Repeat the photometric measurement with 10 ml of the urine thus diluted.
- (8) *Photometric calculation.* When the color is developed in the 500-fold diluted urine the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = 150 \times \frac{D_u}{D_s}$$

When the dilution is other than 500-fold the calculation is:

$$\text{Mg sulfonamide per 100 ml urine} = \frac{750}{V} \times \frac{D_u}{D_s}.$$

D_u is the optical density of the unknown, D_s the density of the standard with 0.3 mg of sulfonamide per 100 ml, V is the ml of fiftyfold diluted urine diluted to 50 ml in the "second dilution."

Note. For determination of ethyl alcohol in urine the procedure is the same as described for blood analysis in paragraph 216.

177. Friedman Test for Pregnancy

a. Collect a morning specimen of urine in a clean container. Sterile precautions are not necessary, but in case of delay the specimen may be preserved by adding one drop of tricresol to 25 ml of urine, or about 0.4 grams of boric acid crystals per 100 ml of urine. Keep the specimen refrigerated after it is received in the laboratory.

b. Filter the urine and test the reaction with litmus paper. If alkaline, make slightly acid by adding a few drops of 10 percent acetic acid.

c. Inject 15 ml of urine warmed to room temperature into the marginal vein of the ear of a virgin female rabbit. (If the specific gravity of the urine is less than 1.020, save a portion and inject 10 ml the next day.) The rabbit should be not less than 17 weeks old and should weigh not less than 1,500 grams. It should be kept isolated from male rabbits for 8 weeks before the test and should be kept in a separate cage for 10 days before the test to avoid any mechanical stimulation of sexual organs that might cause ovulation.

d. After 48 hours the rabbit is killed and the ovaries are examined for the presence of hemorrhagic follicles. One or more hemorrhagic follicles denotes a positive test.

e. If preferred, the rabbit may be anesthetized with ether or intravenous sodium amytal (60 mg. per kg. of body weight) and an operation performed to examine the ovaries instead of sacrificing the animal. Negative rabbits can be used again immediately; rabbits with positive tests can be used after an isolation of 10 to 14 days.

f. Drugs such as aspirin, barbiturates, and quinine, taken by patients, occasionally cause toxicity of the urine for rabbits. The following method may be used to remove such substances as well as other possible contaminants:

- (1) Measure the filtered, acidified urine and add five times the volume of 95 percent ethyl alcohol. Mix well.
- (2) Let stand overnight, decant the supernatant fluid and centrifuge. Pour off the remaining supernatant, and to the sediment add ether equivalent to the original volume of urine. Stir about 10 minutes and centrifuge. Pour off the ether and let the sediment dry.
- (3) Add distilled water to bring to the original volume of urine and shake to dissolve the sediment. If the sediment does not all go into solution, filter. The specimen is now ready to inject into the animal.

178. Quantitative Ascheim-Zondek Test

a. GENERAL. Patients with hydatidiform mole, chorio-epithelioma or certain malignant tumors of the testis excrete increased amounts of follicle-stimulating hormone in the urine. Estimation of the amount of hormone excreted is of assistance in diagnosis and in following the course of these patients after operation or radio-therapy. Females excrete larger amounts of hormone than males and urine specimens from females require dilution for estimation of the quantity of hormone while those from males require concentration.

b. FEMALES. Collect a fresh morning specimen of urine in a clean container and keep in the refrigerator until used. If the specimen must be shipped, add 1 drop of tricresol for each 25 ml of urine. If the urine is alkaline, add 10 percent acetic acid until faintly acid. Filter if cloudy. Prepare 1:2, 1:10, 1:50, and 1:100 dilutions of urine and inject 0.2 ml of each dilution subcutaneously into immature female mice weighing 5 to 8 grams and 20 to 30 days old. The injections are repeated over a 2-day period until 5 injections have been given. It is best to use several mice for each series of doses since some of the mice may die. About 100 hours after the first injection, kill the mice with ether or illuminating gas and examine the ovaries for corpora hemorrhagica, indicated by bright red spots 1 mm in diameter and projecting above the surface. It is best to use a hand lens or a dissecting microscope to examine the ovaries. Report the results in mouse units of gonadotropin per 1,000 ml of urine as indicated by the dosage which gave a positive result. Two ml of undiluted urine (5 doses of 0.4 ml each) indicates 500 mouse units per 1,000 ml; 1 ml of undiluted urine represents 1,000 units; 0.5 ml of undiluted urine represents 2,000 units. One ml of 1:10 dilution is the equivalent of 0.1 ml urine and represents 10,000 units, etc. If the test is positive with the 1:100 dilutions, repeat using higher dilutions.

c. MALES.

- (1) The urine specimen is collected as described above, filtered if cloudy, and made slightly acid with 10 percent acetic acid if alkaline. Part of the specimen is concentrated and part is used unconcentrated.
- (2) Add 100 ml of 95 percent ethyl alcohol to 20 ml of urine, mix, and allow the precipitate to sediment overnight.
- (3) Decant the supernatant leaving about 20 ml. Mix and pour into a centrifuge tube and centrifuge for 5 minutes at 2,000 rpm and pour off the supernatant.
- (4) Add 30 ml of ether, mix by stirring with a glass rod for 10 minutes and again centrifuge for 5 minutes and decant the ether. Distribute the precipitate around the bottom of the tube with a glass rod and allow to dry. Add 4 ml of distilled water and let stand overnight.
- (5) Centrifuge and save the supernatant. Keep in the refrigerator until used. This extract is a 5X concentrate of the urine.
- (6) Six immature, female mice weighing 5 to 8 gm and 20 to 30 days are used in the test.
- (7) The unconcentrated urine should be injected when received without waiting for completion of the concentrations. Keep in the refrigerator between injections.
- (8) Each of 3 mice is injected subcutaneously with 0.1 ml, 0.2 ml, and 0.4 ml, respectively, of unconcentrated urine repeating the injection for a total of 5 doses over a 2-day period.
- (9) Each of 3 other mice receive a similar dosage of the concentrated urine.
- (10) At the end of the 100 hours, kill the mice with ether or illuminating gas and examine the ovaries.
 - (a) *Negative*. The ovaries are small and white.
 - (b) *Positive*. Reaction I consists of follicular ripening with hyperemia and swelling of the follicle. Reaction II consists of follicular hemorrhage.
- (11) Positive tests are reported in mouse units per liter of urine depending upon which mice show a positive reaction. If the 0.1 ml dosage of unconcentrated urine produces follicular hemorrhage, the urine is reported as containing at least 2,000 units per liter. The following chart indicates the value for each urine dosage.

	Mouse	1st Day		2nd Day			Total urine equivalent	Mouse units per liter reaction I and II
		1300	1700	0900	1300	1700		
Unconcentrated Urine	1	0.1	0.1	0.1	0.1	0.1	0.5	2,000
	2	0.2	0.2	0.2	0.2	0.2	1.0	1,000
	3	0.4	0.4	0.4	0.4	0.4	2.0	500
Concentrated Urine	4	0.1	0.1	0.1	0.1	0.1	2.5	400
	5	0.2	0.2	0.2	0.2	0.2	5.0	200
	6	0.4	0.4	0.4	0.4	0.4	10.0	100

CHAPTER 5

EXAMINATION OF GASTRIC CONTENTS

Section I. PHYSICAL EXAMINATION

179. Macroscopic

a. AMOUNT. The capacity of the average adult's stomach is approximately 1,500 ml. The fasting residuum varies up to 100 ml but is rarely over 50 ml unless obstruction is present.

b. EMPTYING TIME. Emptying time varies greatly, but the normal stomach is usually empty within 7 hours after a light meal.

c. APPEARANCE AND ODOR. The normal fasting residuum may be thin, clear, and colorless, but usually it is turbid, grey, or bile tinged, with a greater or lesser admixture of saliva and mucus. Occasionally it is thin but usually it is mucoid or syrupy. Blood may appear as such, but usually small amounts, changed by the stomach acid, give a light brown to dark chocolate tint depending on the quantity. Clear gastric juice has practically no odor; if much mucus or saliva is present there is a slightly sour, musty smell. Only very rarely is the odor foul.

180. Microscopic

Microscopic examination of gastric contents is unnecessary as a routine. Materials that may be looked for on occasion are blood, food particles, bits of tissue or tumor, foreign body material, and tubercle bacilli that have been swallowed. Very rarely parasites may be found, probably swallowed with food or water.

Section II. TESTS OF GASTRIC SECRETION

181. Basal Gastric Secretion

a. PROCEDURE. The patient should be at rest (sitting or reclining) having taken no food or fluid for 12 hours. A duodenal tube is passed for a sufficient distance to allow its tip to reach the most dependent part of the stomach. The position of the tube is maintained by strapping it to the angle of the mouth with adhesive. The patient is urged not to swallow saliva, and this point is emphasized throughout the test. A vessel is furnished for expectoration. As soon as the tube is in place the fasting contents are withdrawn with a syringe. A 50-ml Luer syringe is most satis-

factory. Continuous suction is kept up, and the material obtained is segregated in graduated tubes in portions obtained over successive 10-minute periods. Aspiration is kept up for 1 hour, when the test is terminated. After a few periods, the flow of juice usually reaches a nearly steady level. The amount of juice obtained during a 10-minute period after this point is reached, and its acidity, are taken as the basal secretory values for that person. Done in this way remarkably constant results are obtained on the same subject on successive days.

b. RESULTS. The values for basal secretion in healthy people are as follows: 10-minute secretory volume, 5 to 25 ml; total acidity, 0° to 110° ; and free acid, 10° to 30° less than the total. The regurgitation of any large amount of bile interferes with the test, which must then be repeated on another day. As a rule the measurement of basal secretion suffices. However, if no free acid is present, one may proceed to the histamine test.

182. Histamine Test

If the basal test yields no free acid, histamine may be given to see whether a true anacidity exists. Histamine is useful in stimulating the greatest flow of juice of highest acidity of which the stomach is capable.

a. PROCEDURE. After the fasting secretions have been collected over two or three 10-minute periods and have reached a minimum, 1 ml (1 mgm) of histamine phosphate is injected subcutaneously to stimulate gastric secretion. This dose is adequate; although a wheal is produced at the site of injection and at times the patient complains of a hot, flushed feeling in the face and neck, noteworthy constitutional symptoms rarely occur. After the injection of histamine, aspiration of gastric juice is continued over four to six 10-minute periods. A certain amount of practice is necessary before the procedure can be carried out satisfactorily. At first, owing to the alternating phases of tone and relaxation to which the fasting stomach is subject, it is difficult to make continuous aspiration and there is a tendency to traumatize the mucosa. After a little experience one develops a tactile sense that renders the procedure simple. If there is marked bile staining, the test must be discarded and repeated on another day. The aspirated material is normally a clear, limpid, or slightly opalescent fluid containing more or less mucus, which can be readily removed by centrifugation. Such juice is suitable for chemical tests of all sorts.

b. RESULTS.

- (1) *Volume.* The largest amount of gastric juice obtained during any single 10-minute period after histamine

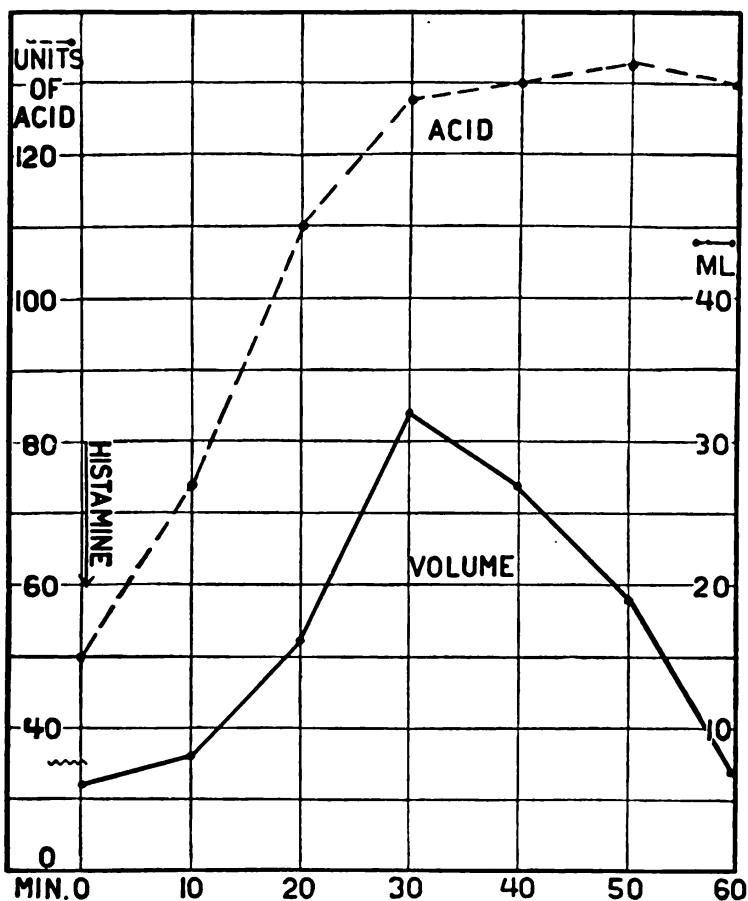


Figure 17. Curve of acidity and volume of secretion in a normal person after histamine stimulation. (The total secretion was collected over 10-minute periods.)

varies in normal people from 5 to 50 ml. The latter value is only very rarely exceeded. However, in the majority of cases maximum 10-minute secretion is from 15 to 40 ml.

- (2) *Acidity.* The highest acidity (total) obtained during the course of the test varies from 0° to 160° , but in the majority of normal people it lies between 80° and 130° . (See fig. 17.)

Section III. CHEMICAL ANALYSIS

183. General

Examine all specimens in the order collected.

184. Technic

a. REAGENTS.

- (1) *Töpfer's reagent.* Dissolve 0.5 gm of p-dimethylaminoazobenzene in 100 ml of 95 percent ethyl alcohol.
- (2) *0.1 N sodium hydroxide.*
- (3) *Phenolphthalein indicator.* Dissolve 0.05 gm of phenolphthalein in 100 ml of 50 percent ethyl alcohol.

b. PROCEDURE.

- (1) "*Free hydrochloric acid.*" Place 5 ml of gastric juice (filter, if necessary) in a small beaker. Add a drop or two of Töpfer's reagent. If the pH is low enough to indicate what is called arbitrarily "free HCl," the solution turns a bright red color. Add 0.1 N sodium hydroxide solution from a burette until the red color turns salmon pink. The number of milliliters of hydroxide used multiplied by 20 gives the "free-acidity" value in degrees. One hundred degrees is the equivalent of 0.1 N HCl.
- (2) "*Total acidity.*" After the completion of procedure (1), add 1 drop of the phenolphthalein indicator, and titrate with 0.1 N sodium hydroxide solution from a burette until there is produced a faint pink color that persists for 2 minutes. The total number of ml of alkali used in (1) and (2) multiplied by 20 gives the "total-acidity" in degrees. This is merely a measure of total buffer effect arbitrarily stated.

c. RESULTS. With the histamine test, "free HCl" is from 5 to 30° less than the "total acidity." This indicates that the clear gastric juice obtained after histamine has only slight buffer value.

185. Occult Blood (Guaiaac Test)

a. REAGENTS.

- (1) Hydrogen peroxide 3 percent.
- (2) Acetic acid, glacial reagent.
- (3) *Guaiaac solutions.* Prepare a fresh solution each time the tests are performed by dissolving 0.5 gm of powdered gum guaiac in 30 ml of 95 percent alcohol.

b. PROCEDURE FOR GASTRIC JUICE.

- (1) *Direct test.* Add 1.0 ml of hydrogen peroxide to 1.0 ml of the fresh guaiac solution. Place 2.0 ml of the material for analysis in a test tube and make strongly acid with acetic acid. Add 0.5 ml of the peroxide-guaiac solution and observe for the appearance of a violet or blue color. The intensity of the color and the speed with which it

develops are a rough measure of the amount of blood present. An excess of the peroxide-guaiac interferes with the delivery of the test solution.

- (2) *Confirmatory test.* If fat is present, make the test material slightly alkaline with sodium carbonate or sodium hydroxide solution. Extract in a separatory funnel with an equal amount of ether. Discard the ether extract. Make the residue slightly acid with acetic acid and again extract with ether. Evaporate the ether to dryness on a water bath. Dissolve the residue in a few drops of water. Add a drop of guaiac solution and a drop of hydrogen peroxide and observe for the blue color.

c. PROCEDURE FOR FECES.

- (1) *Ether extraction test.* Make a thin fecal suspension in approximately 5 ml of water. Shake with 5 ml of ether to remove fat. Discard the ether extract. Acidify the residue with acetic acid and again extract with 5 ml of ether. Evaporate the ether extract and test as above.
- (2) *Direct test.* Smear a small portion of feces on a microscopic slide or a piece of blotting paper. Acidify with a few drops of glacial acetic acid and add 2 or 3 drops of the guaiac-peroxide solution. Observe for the development of a blue color.

186. Other Tests

Tests for lactic acid, pepsin and nitrogen give no useful clinical information and are no longer advised in routine work. For total chloride use a 1 ml sample of gastric juice and apply the method described for plasma chloride. (See par. 209.)

CHAPTER 6

CHEMICAL ANALYSIS OF BLOOD AND SPINAL FLUID

Section I. PREPARATION OF BLOOD, PLASMA, AND SERUM FOR QUANTITATIVE CHEMICAL ANALYSIS

187. Handling Blood and Treating With Anticoagulants

a. DRAWING AND TRANSFER OF BLOOD.

- (1) The object in preparing blood for chemical analysis is to bring to the analysis samples of blood, plasma, or serum in which the substances to be determined have exactly the same concentrations as in the circulating blood. The technique for drawing blood has been described in section X, chapter 1.
 - (a) In transfer from syringe to tubes, the blood should be handled gently to avoid hemolysis. Do not squirt the blood from syringe to tube, especially not through a fine needle as this is apt to cause hemolysis. Instead, lower the tip of the needle to the bottom of the tube and expel the blood gently, raising the tip of the needle so that it remains just under the rising surface of the blood. If the needle does not reach to the bottom of the tube, let the blood drain down the side of the tube, and not fall through the air.
 - (b) The tubes or flasks receiving the blood should be of such size that the blood will fill them from one-third to two-thirds full. If the blood, or serum or plasma from it, is to be shipped, or for other reasons not analyzed on the day drawn, the blood should be drawn under the sterile precautions described in section X, chapter 1, and the receiving tubes or flasks should be sterile.
- (2) If the blood is kept at ordinary room temperature analysis should be begun, or serum, plasma, or protein-free filtrate should be separated, within 1 hour after the blood is drawn, except in the special cases where preservation for shipment is permissible. (See tables XIV and XV.) Avoidance of delay is specially important in sugar analyses. If the blood is at once chilled in ice water and kept in a refrigerator near 0° C., it may be kept for several hours.

b. TREATMENT WITH OXALATE.

(1) *Preparation of tubes or flasks with potassium oxalate.*

(a) Because of its ready solubility, potassium oxalate is more frequently used as anticoagulant than other oxalates. One mg of potassium or sodium oxalate per ml of blood suffices to prevent clotting; more than 2 mg per ml begins to cause hemolysis, and to alter measurably the distribution of water and electrolytes, notably the chloride and bicarbonate (alkali reserve) between cells and plasma. Hence, the vessels to receive blood are coated with oxalate measured to provide between 1 and 2 mg for each ml of blood. It is desirable to prepare sterilized tubes and to coat them in advance with the oxalate in films on the walls; in this form, the oxalate dissolves instantly, and a minimal amount prevents coagulation.

(b) For each ml of blood to be received, 0.01 to 0.02 ml of a 10 percent neutral potassium oxalate solution is pipetted into the receiving tubes or flasks. For most purposes, two sets of receivers will suffice. One set, for 6 to 10 ml portions of blood, receives in each vessel 0.10 ml of 10 percent of potassium oxalate solution; the other set, for 3 to 5 ml of blood, receives 0.05 ml of the same solution. The oxalate solution is distributed in a thin film about the lower part of the vessel, and is dried, by leaving the vessel in a desiccator over calcium chloride, or in an incubator at 38°, or in an oven at less than 80° C. Higher temperature decomposes the oxalate into carbonate.

(2) *Other oxalates, including Heller and Paul's mixture.* Lithium or sodium oxalate may be used in place of the potassium salt. Ammonium oxalate, because of its NH_3 content, cannot be used when urea or Kjeldahl determinations are to be done, hence is excluded from general use. Heller and Paul's mixture (2 parts potassium oxalate and 3 parts ammonium oxalate) has the advantage that it changes the cell volume but little; it is desirable in the special cases that hematocrit determinations are to be done, or that the copper sulfate method for hemoglobin, hematocrit, and plasma proteins is to be applied.

c. ANTICOAGULANTS OTHER THAN OXALATE.

(1) Heparin 0.2 mg per ml of blood is excellent for all purposes. It is the one anticoagulant which provides a

plasma that can be used as well as serum for calcium determination.

- (2) Sodium citrate, 5 mg per ml can be used when whole blood is to be analyzed, but is less desirable than oxalate when plasma is to be analyzed, because the citrate disturbs more the distribution of water and electrolytes between cells and plasma.
- (3) Sodium fluoride, 10 mg per ml of blood, will prevent not only coagulation, but also loss of sugar by glycolysis, and, furthermore, appears to stabilize uric acid. Fluoride also acts as a bacteriostatic agent, and with 1 mg of thymol per ml of blood will prevent bacterial changes for a week. It is recommended only when whole blood must be shipped for creatinine, nonprotein nitrogen, or sugar analysis. (See table XIV.) It cannot be used if urea is to be determined, as it inhibits the action of urease.

188. Separations of Serum and Plasma

a. SERUM.

- (1) Blood serum is blood plasma without the fibrin. The blood is transferred to a round bottomed tube without anticoagulant. Let the tube stand at room temperature a short time until the clot has formed, then chill in ice water and place in the refrigerator. When the clot has contracted, gently free the top part from the tube if it sticks, then centrifuge. Transfer the serum to a clean, dry tube. If serum is to be shipped for analysis, use sterile precautions.
- (2) If a centrifuge is not available, clear serum may be obtained by placing the tube containing the clotted blood in a nearly horizontal position, forming a long slanting surface. After clotting is complete, chill thoroughly, then place the tube upright in a refrigerator overnight. In the morning, pour off the clear serum from the side of the tube opposite the slant.

b. PLASMA. Oxalated or heparin-treated blood is centrifuged for 10 to 20 minutes at 2,000 to 3,000 rpm.

189. Preservation of Blood

If blood cannot be analyzed immediately, precautions must be taken to prevent changes in the concentration of substances to be

determined. The changes to be prevented and the means to avoid them are chiefly the following.

a. DECREASE IN VOLUME BY EVAPORATION. Blood, plasma, serum, and filtrates are to be kept in *stoppered* vessels, which are opened only momentarily for withdrawal of samples. During centrifugation, tubes are covered with rubber caps; centrifuging for even 1 minute in an open tube can cause a significant evaporation, measurable by weighing the tube before and after the centrifugation.

b. ENZYMIC CHANGE. As soon as the blood is shed, enzymes in the cells begin to turn the glucose into lactic acid, to split free phosphoric acid from organic phosphates, to consume O_2 , and to produce CO_2 . These changes affect not only cell composition, but by diffusion also the glucose, alkaline reserve, and phosphate content of the plasma. The acidifying changes also cause the cells to swell, increasing the protein concentration in the plasma, while the plasma chloride is decreased by diffusion into the cells. *For all plasma and serum analyses, separation from the cells should be carried out soon, preferably within 1 hour after the blood is drawn.* For sugar and nonprotein nitrogen analyses, the whole blood can be preserved by adding fluoride, which retards enzymic changes.

c. BACTERIAL CHANGES. The changes produced by bacteria are unpredictable. They include destruction of the sugar, hydrolysis of the urea to ammonium carbonate, and digestion of the proteins. To prevent these changes blood, serum, and plasma, if not analyzed on the same day when drawn, are handled with sterile technic. When serum can be used, it is preferable to plasma because the oxalate used to obtain the plasma cannot be completely sterilized, although the 10 percent solution is somewhat bactericidal. Similarly, when defibrinated whole blood can be shipped it is preferable to oxalated blood because the defibrinated blood can be sterile. Addition of 10 mg of sodium fluoride and 1 mg of thymol per ml of blood retards the growth of bacteria, but no preservative is satisfactory if gross bacterial contamination has occurred.

d. INTERCHANGE OF SUBSTANCES BETWEEN CELLS AND PLASMA.

- (1) In blood as drawn none of the substances analyzed has identical concentrations in plasma and cells; the proteins and organic phosphates are entirely different in the two phases; potassium is almost entirely in the cells, sodium

in the plasma; chloride and CO_2 are almost twice as concentrated in plasma as in cells; amino acids are twice as concentrated in the cells. Of the plasma constituents ordinarily determined, only urea, sugar, creatine, and uric acid have nearly enough the same concentrations in cells and plasma *in vivo* to give their determination in whole blood acceptable clinical significance. It is essential to keep the other substances confined within cells and plasma, as when drawn, until the plasma is separated for analysis, or the results will not indicate plasma concentration *in vivo*. The forces that keep cell and plasma constituents apart begin to break down soon after blood is drawn, and the constituents of the two phases begin to mix. To prevent this, as well as the effects of cellular enzymes, follow the precautions of paragraph 187 to prevent hemolysis, and separate the serum or plasma soon after the blood is drawn, preferably within 1 hour.

- (2) In table XIV are summarized the amounts of blood required for different analyses and other data on conditions that must be observed in preparing blood, plasma, or serum for the analyses.

Table XIV. Specifications for Handling Samples of Whole Blood for Analysis

Analysis	Ml of blood to collect	Can be shipped or not for analysis	Maximum time allowable between drawing and analysis	Precautions
Alcohol	10	Yes	72 hours	Sodium citrate 50 mg and sodium fluoride 75 mg. per 10 ml.
Carbon monoxide.	5 to 10	Yes	Undetermined	Avoid escape of CO by storing in closed vessel with little air space.
Hemoglobin and hematocrit by copper sulfate gravity method.	0.2 to 5.0. 1 ml suffices for plasma proteins also.	No. Can be analyzed at bedside.	Few hours	Avoid more oxalate than directed. Avoid hemolysis.
Nonprotein N.....	5	Yes, with fluoride preservative.	1 week with fluoride.	Shipment of fluoride blood permissible, but better to precipitate proteins and ship filtrate.
Sugar	5	Yes, with fluoride.	1 week with fluoride. One hour without F.	Either add fluoride or, better, precipitate proteins at once after drawing blood.
Sulfonamides	3 to 7	Yes	No. definite.....	
Urea	5 to 10.....	Yes, defibrinated sterile.	1 to 2 days.....	Do not use fluoride; it inhibits urease action.

Table XV. Specifications for Handling Samples of Plasma or Serum for Analysis

Analysis and material used for it	Ml of blood to collect	Do plasma and serum stand shipment for this analysis?	Maximum time allowable between separation from cells analysis	Precautions
Albumin, total protein, and A/G ratio. Plasma.	8	Yes	2-3 days.....	Avoid hemolysis. Avoid high temperature during transportation.
Bilirubin. Serum	5	No	2 hours	Avoid hemolysis.
Calcium. Serum or heparin plasma	5	Yes	Indefinite if sterile.	
Carbon dioxide capacity. Oxalate plasma.	10	Yes	24 hours.....	Avoid permitting escape of CO ₂ from whole blood by streaming or agitation in air before centrifuging. Use neutral oxalate.
Chloride. Plasma or serum.	2-5	Yes	Indefinite	Avoid excess oxalate. Use same precautions as for CO ₂ capacity.
Cholesterol, total. Plasma or serum.	5	Yes	Indefinite	
Cholesterol, esterified. Plasma or serum.	5	No	2 hours at 20°.	Plasma or serum may be kept 1 or 2 days in ice box. Avoid hemolysis.
Creatinine. Plasma or serum.	5	Yes	1 week with fluoride, less without.	
Phosphate, inorganic. Plasma or serum.	8	Yes	Trichloroacetic filtrate lasts indefinitely.	Avoid hemolysis and delay between drawing and centrifuging.
Phosphatase Serum.	10	No	1 hour at room temperature; 24 hours in refrigerator.	Same as for inorganic phosphate.
Proteins by copper sulfate gravity method. Plasma or serum.	1-5. Suffices also for hemoglobin and hematocrit.	Yes	2-3 days.....	Avoid more oxalate than specified. Avoid hemolysis.
Uric acid. Plasma or serum.	5	Yes	1 week with fluoride.	

190. Preparation of Protein-free Blood Filtrate (Folin and Wu, J. Biol. Chem., 38, 81 (1919); 51, 419 (1922); Haden, J. Biol. Chem., 56, 469 (1923))

a. REAGENTS.

- (1) *Sodium tungstate* ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$), 10 percent solution. Ten grams of $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ per 100 ml of solution. If

the solution is too acid or too alkaline, it will not precipitate proteins completely. Pipette a 10-ml portion into a flask and add a drop of phenolphthalein indicator. Titrate with 0.1 *N* H₂SO₄ or NaOH, whichever is required to bring the color to slight pink. If more than 0.4 ml of 0.1 *N* acid or alkali is required, add to the main solution of tungstate, per 100 ml, a volume of 1.0 *N* sulfuric acid or NaOH equal to the volume of 0.1 *N* required in the test titration. The Na₂WO₄ solution is 0.3 molar.

- (2) *Sulfuric acid, N/12 solution.* Dilute one volume of 1 *N* sulfuric acid with 11 volumes of water.
- (3) *Sulfuric acid, N/12, with 0.25 percent benzoic acid.* Per 100 ml of the *N/12* acid, add 0.25 gm of benzoic acid and dissolve.
- (4) *Sulfuric acid, 10 percent.* Ten ml of concentrated H₂SO₄ to 100 ml.
- (5) *Sulfuric acid, 0.67 N.* Dilute 67 ml of 1 *N* sulfuric acid to 100 ml.

b. PROCEDURE.

(1) *Whole blood.*

- (a) Pipette the sample of blood or plasma into a volumetric flask of capacity equal to 10 times the volume of the sample. Add the *N/12* sulfuric acid until the flask is about three-fourths full. Mix the acid and the blood by gentle whirling. Then add a volume of 10 percent tungstate equal to the volume of the blood sample, fill the flask to the mark with the *N/12* acid, stopper, and mix by inverting 10 times. (If the filtrate is to be shipped for analysis, use the *N/12* H₂SO₄ with added benzoic acid, which acts as a preservative.)
- (b) Instead of using a volumetric flask, one may pipette the sample of blood or plasma into an Erlenmeyer flask, add 8 volumes of *N/12* sulfuric acid, mix by rotation, and add 1 volume of 10 percent tungstate.
- (c) When whole blood is thus treated, the mixture should change from pink to brown. If this change does not occur in a few minutes, the coagulation is incomplete, usually because too much citrate or oxalate has been added previously. If the proper color change fails to occur, pour the mixture into an Erlenmeyer flask and add 1 drop of 10 percent sulfuric acid for each ml of blood present, mix by whirling, and note whether the color change occurs. If it does not, repeat the addition.

- (d) Either centrifuge or filter the mixture. Centrifugation yields somewhat more filtrate. Cover the centrifuge tube with a cap to prevent evaporation during centrifugation.
 - (e) For filtration, use a dry filter large enough to hold the entire mixture, and cover the funnel with a watch glass. If the first portion of filtrate is not clear, return it to the filter.
 - (f) As soon as filtration is complete, place the filtrate in a stoppered flask to prevent evaporation.
- (2) *Spinal fluid.* Prepare a 1:5 mixture for protein-free filtrate in place of the 1:10 above. To each volume of spinal fluid add 3 volumes of distilled water, 0.5 ml of 10 percent sodium tungstate and 0.5 ml of 0.67 *N* sulfuric acid. Allow to stand 10 minutes and then filter. Normal values for spinal fluid are 40 to 70 mg glucose per 100 ml.

Section II. CHEMICAL ANALYSIS

191. Nitrogen by Microkjeldahl Analysis, Titrimetric¹

a. GENERAL.

- (1) In Kjeldahl's method organic nitrogen is converted into ammonia by heating with concentrated sulfuric acid, with addition of catalysts to accelerate the conversion and make it quantitative. In the present procedure the catalysts are mercuric and potassium sulfates. The ammonia formed by the digestion is set free by alkali and is driven by a current of air into boric acid solution by the procedure described for determination of urea and ammonia in urine (par. 172), the ammonia caught in the boric acid solution being titrated with standard sulfuric acid solution. With the alkali used to set the ammonia free, thiosulfate is added to prevent the mercuric oxide from holding back ammonia during the aeration.
- (2) Compared with the colorimetric procedure described in paragraph 192, the titration method has the advantage that it requires less skill in handling the digestion with sulfuric acid, and that its results are likely to be somewhat more exact. The colorimetric method, on the other hand, has the advantages of being quicker, and of requiring only 0.05 to 0.2 mg of nitrogen, compared with 0.5 mg or more for the titration method.

¹Sobel, *Journal of Biological Chemistry*, 118, 443 (1934) modified by Hiller (unpublished).

b. APPARATUS.

- (1) The heavy-walled 32- by 200-mm test tubes, the racks, etc., described for the determination of urea and ammonia in urine. (See par. 172.)
- (2) Pyrex test tubes, 25 by 200 mm or 20 by 150 mm, for the digestions.
- (3) A burette, 10 or 25 ml.
- (4) Chips or beads to prevent bumping during digestion. The silica chips described for the colorimetric method (par. 192a) may be used.
- (5) Digestion rack to hold the Pyrex tubes during digestions. Microburners with wind shields provide heat for the digestions. The mouths of the tubes should rest in holes in a lead or glass pipe which is connected with a good suction to carry away fumes of sulfuric acid.

c. REAGENTS.

- (1) Concentrated sulfuric acid, reagent grade.
- (2) Potassium sulfate, pulverized, reagent grade.
- (3) Mercuric sulfate solution. Pour 12 ml of concentrated sulfuric acid with stirring into 100 ml of water. Add 10 gm of mercuric oxide and warm until dissolved.
- (4) Alkaline thiosulfate solution. Dissolve 240 gm of sodium hydroxide in 560 ml of water and cool. Dissolve 50 gm of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) in 80 ml of water. Mix the two solutions.
- (5) Boric acid, 4 percent.
- (6) Brom cresol green indicator, 0.1 percent alcoholic solution. Dissolve 100 mg of brom cresol green in 100 ml of 95 percent alcohol.
- (7) Caprylic alcohol.
- (8) Standard sulfuric acid solution, 0.01414 N. From a 25-ml burette deliver 14.14 ml of standardized 1 N sulfuric acid into a 1-liter volumetric flask, dilute to volume with water, and mix.

d. PROCEDURE.

- (1) *Digestion.* Into one of the Pyrex digestion tubes measure a sample containing 0.5 to 3.0 mg of nitrogen. Add 1 ml of concentrated sulfuric acid, 0.5 gm of K_2SO_4 , 0.5 ml of mercuric sulfate solution, and 2 or 3 antibump chips. Place the tube in a slanting position, with the mouth in a flue or other device to carry away fumes. Heat with the tip of the shielded microburner until water has been driven off and the charred solution has become clear. Then continue the heating of the cleared solution for 30 minutes longer. If any particles of carbon

are noted on the wall of the tube above the digestion fluid, turn the tube so that the particles are brought into the fluid before the final 30-minute period is begun. During the entire digestion the mixture must be definitely boiling.

- (2) *Aeration of ammonia.* The digest is allowed to cool (but not to stand till it hardens), then is washed into one of the large aeration tubes with 10 ml of water, which is used in 4 or 5 portions for the washing. A receiving tube for the ammonia is prepared with 25 ml of 4 percent boric acid, 0.1 ml of indicator solution, and a drop of caprylic alcohol, as described in paragraph 172, and the connections are made ready for the aeration. Three drops of caprylic alcohol are added to the tube with the digest, the connections are completed, and a slow current of air is drawn through the pair of tubes. From a graduated pipette 7 ml of the alkaline thiosulfate solution 10 *N* NaOH solution is run into the tube that contains the digest, by way of the glass tube that serves as air inlet. The current of air draws the alkali in and mixes it with the acid. The aeration set is then connected with the wash bottle and the aeration is carried out as described in paragraph 172. A dozen or more pairs of tubes can be aerated in one train together.
- (3) *Titration.* The titration is carried out as described for determination of urea and ammonia (par. 172) except that the 0.01414 *N* sulfuric acid is used for the present titration. The burette should have a fine tip attached so that drops not larger than 0.02 ml can be delivered.
- (4) *Blank.* Blank analyses are done in which the digestion mixture is heated and the digest is alkalized, and aerated as in the analyses, and any ammonia caught in the boric acid is titrated.
- (5) *Calculation.* $Mg\ N\ in\ sample = 0.2\ (A - B)$.
A represents the ml of 0.01414 *N* H₂SO₄ used in titrating the NH₃ yielded by the unknown, *B* the ml of 0.01414 acid used in titrating the blank on the reagents. If the yield of NH₃ were 100 percent, the normality of H₂SO₄ that would be used, in order to apply the factor 0.2 in the calculation, would be 0.01428, instead of 0.01414 *N*. However, the method described yields with uniformity 99 percent of the theoretical ammonia. In order to correct for this deficit of 1 percent, the standard H₂SO₄

solution is made 1 percent less than theoretical in concentration.

192. Nitrogen by Microkjeldahl Analysis, Colorimetric²

a. GENERAL. The ammonia formed by digestion with sulfuric acid and catalysts (par. 191) is estimated from the color obtained by adding Nessler's solution to the diluted digest. It is essential to obtain complete digestion, with quantitative yield of ammonia, and at the same time to avoid the use of reagents which may produce turbidity, or the use of prolonged digestion, which might produce turbidity from silicate detached from the glass digestion vessel. These objects are attained by using potassium persulfate to finish quickly the oxidation of the organic matter. The persulfate is not added till the final stage of the digestion; if it were added at the beginning of the digestion of a water solution, it might be entirely decomposed by the hot water ($2\text{K}_2\text{S}_2\text{O}_8 + 2\text{H}_2\text{O} \rightarrow 4\text{KHSO}_4 + \text{O}_2$) before oxidation of the organic matter was completed. When the digested material is protein, the nitrogen obtained by this method is only 97 percent of the total, and a factor of 1.03 is used in the calculation to correct for the 3 percent deficit. The relative advantages of the colorimetric method compared with titrimetric are discussed in paragraph 191.

b. REAGENTS.

- (1) *Digestion mixture.* Mix concentrated reagent grade sulfuric acid with an equal volume of 0.3 percent water solution of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).
- (2) *Potassium persulfate* ($\text{K}_2\text{S}_2\text{O}_8$), 2.5 percent water solution.
 - (a) The solution is prepared without heating in order to avoid decomposition of the persulfate. The solution can be kept in an ice box below 5°C . for 6 months, or at room temperature for about 1 month, without decomposition of more than 10 percent of the persulfate, which is permissible.
 - (b) The persulfate used must be of a special, ammonia-free grade, with less than 0.01 mg of ammonia nitrogen per gram. Persulfate containing somewhat more ammonia can be purified by recrystallization. To recrystallize 100 gm, heat 250 ml of water to boiling in a 500 ml Erlenmeyer flask, remove the flask from the flame, add the persulfate, and stir until dissolved. Do

² Wong, *Journal of Biological Chemistry*, 55, 431 (1923), modified by Buell and by Archibald (unpublished).

not heat after adding the persulfate, or decomposition will occur. As soon as solution is complete, cool under the tap, then in an ice box. Filter the crystals on a Buchner funnel (par. 119c), and dry in a desiccator or in air at room temperature.

- (3) *Nessler's solution.*³ Dissolve 45.5 gm (0.1 mole) of mercuric iodide (HgI_2 , N.F. grade) and 34.9 gm (0.21 moles) of potassium iodide in 500 ml of water in a 1-liter volumetric flask. Then add 200 ml of 10 molar KOH solution (2 moles), mix, dilute to 1 liter, and mix again. If any precipitate is present, let it settle, and decant the clear solution.
- (4) *Standard solution of ammonium sulfate containing 0.1 mg of nitrogen per ml.* In a 1-liter volumetric flask dissolve 0.4717 gm of reagent grade $(\text{NH}_4)_2\text{SO}_4$ and make up to volume. Before diluting to volume with water, add 0.5 ml of concentrated sulfuric acid to improve the keeping qualities of the standard. This standard lasts indefinitely.

c. APPARATUS.

- (1) Pyrex test tubes, 25 by 200 mm, each marked by a complete etched circle at the level indicating 20 ml.
- (2) Silica chips, to prevent bumping during digestion. A rod of "vitreosil" of about 5 mm diameter is broken into pieces about 5 mm long. After repeated use these chips may become ineffective. Their antibump efficiency can be restored by heating them overnight in an oven at 105° C. or higher. The vitreosil chip used in the digestion may be replaced by two Pyrex beads, but they are less efficient.
- (3) Colorimeter or photometer.

d. PROCEDURE.

(1) *Digestion.*

- (a) In one of the marked test tubes place a sample containing 0.05 to 0.20 mg of nitrogen. Add one of the silica chips and 0.30 ml of the digestion mixture. Heat the tube in a slanting position over a microburner which is shielded from drafts, until the water is driven off and the residue just begins to char. Usually 2 to 3 minutes suffice to drive off the water and start charring of the residue. Then allow to cool for 30 seconds, and add slowly drop by drop 0.4 ml of the 2.5 percent persulfate solution, letting the drops fall on the wall

³ Vanselow, Industrial Engineering and Chemistry, Analytical Edition, 12, 516 (1940).

in the upper part of the slanting tube and run down into the digestion mixture. The mixture will begin to boil on contact with the first drop of persulfate solution, and will continue to boil during the addition. Insert a small funnel in the top of the tube to prevent escape of SO_3 fumes or volatilized ammonium sulfate, and resume heating for 1 minute, and as much longer as may be necessary to make the digest become entirely clear. Remove from burner for 30 seconds and add 2 drops more of the persulfate solution through the funnel, with the test tube on a slant. Then finish the digestion by heating 1 minute more.

- (b) The heating should be vigorous enough to make white fumes appear in the lower part of the tube, but not so vigorous that more than traces of the fumes escape from the tube. Too vigorous or too prolonged heating may cause loss of ammonium sulfate by volatilization.
 - (c) If no vitreosil or Pyrex chips are available to prevent bumping, one may digest without them by holding the test tube in a holder, and whirling the contents of the tube about its bottom during the digestion. A regular Bunsen burner may be used instead of a microburner, the bottom of the tube being shaken with a rotary motion in the tip of the flame. Digestion is somewhat quicker by this technic than when the tube is heated in a stationary position with a microburner.
- (2) *Nesslerization.* After the digest has cooled, add water, at first through the funnel, then down the sides of the tube so that all parts of the wall are washed down. Dilute to the 20 ml mark and mix by a rotary motion. Add 5 ml of Nessler's solution from a burette, and mix again. A clean, footed rod may be used, instead of rotation, for mixing.

e. MEASUREMENT IN COLORIMETER.

- (1) Prepare a standard solution by digesting and Nesslerizing 1 ml of the standard ammonium sulfate solution, as described above. Use of a standard thus prepared corrects for traces of nitrogen that may be in the reagents. The standard is Nesslerized at the same time as the unknown. After allowing 5 minutes or longer for development of color, compare unknown and standard in the colorimeter.

Calculation:

$$\text{Mg N in sample} = 0.1 \times \frac{S}{U}.$$

U is the reading of the unknown, S is the standard.

If the digested material is *protein* the factor 0.103 is substituted for 0.1, because the digestion yields only 97 percent of the NaNH_3 . The calculation then is:

$$\text{Mg N in sample} = 0.103 \times \frac{S}{U}.$$

- (2) If the unknown contains less than 0.05 or more than 0.20 mg of nitrogen, and accuracy is necessary, repeat the analysis with a sample of size within these limits, preferably as near 0.1 mg as possible.
- (3) Use of a color filter transmitting light of about 500 millimicrons wave length facilitates the reading.

f. MEASUREMENT IN PHOTOMETER.

- (1) The blank is prepared by digesting and Nesslerizing the reagents. The transmittance or optical density curve is prepared from standard solutions containing 0.05, 0.10, and 0.20 mg of nitrogen which are digested in the same manner as the samples. The solution follows Beer's law if light of a sufficiently narrow band of wave lengths is used. Any wave length between 500 and 450 millimicrons may be employed, but it is particularly important that exactly the same band of wave lengths be used for unknown and standard. There is no plateau or maximum of optical density in the curve of wave length versus density in the visible spectrum; the density increases rapidly in a nearly linear manner as the wave length shortens from 500 to 450, and is about twice as great at 450 as at 500. Hence a difference of 5 millimicrons in wave length can make a difference of 10 percent in density. Especially for dilute solutions, light of wave length 450 has over 500 the advantage of greater absorption. Length shorter than 450 can not be used because the optical density of the reagents becomes appreciable.
- (2) When maximal precision is desired it is advisable to digest a standard solution with each series of analyses and check the slant of the optical density curve, or the k of the calculation formula.
- (3) The blank solution prepared from the digested reagents is used to set the zero of the photometer.

Calculation:

$$Mg\ N\ in\ sample = 0.1 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown, D_s the density of the standard with 0.1 mg of nitrogen.

If the analyzed material is protein, replace the factor 0.1 by 0.103.

193. Nonprotein Nitrogen (NPN) of Blood or Plasma

a. REAGENTS AND APPARATUS. Those used for precipitation of proteins with tungstic acid (par. 190) and for microkjeldahl analyses. (See pars. 191 and 192.)

b. PROCEDURE. Precipitate the proteins in blood or plasma as described in paragraph 190. Determine the nitrogen in aliquot parts of the filtrate as described in paragraph 191 or 192. For colorimetric nitrogen determination (par. 192) 3 ml of the filtrate is usually used, but in analyses of uremic blood it may be necessary to use only 1 ml. For titrimetric determination (par. 191) 5 ml of filtrate of either normal or uremic blood is used as the sample.

c. CALCULATION.

$$Mg\ NPN\ per\ 100\ ml\ blood\ or\ plasma = Mg\ N\ in\ sample \times \frac{1,000}{V}$$

V is the ml of blood or plasma filtrate used as the sample for the nitrogen determination, 1 ml of the filtrate representing 0.1 ml of blood or plasma.

194. Total Protein, Albumin, and Globulin of Plasma⁴

a. GENERAL. The globulins, including the fibrinogen, are precipitated by Na_2SO_4 in 22 percent concentration. In the filtrate the nonprotein nitrogen plus albumin nitrogen is determined by microkjeldahl. The total nitrogen and NPN of the plasma are also determined, and from the three analyses the albumin and globulin are calculated. If the same procedure is applied to serum, the globulin value obtained does not include the fibrinogen.

b. REAGENTS.

(1) The *reagents* for either the titrimetric or the colorimetric *microkjeldahl* method (par. 191 or 192) and for the *tungstic acid precipitation of proteins* (par. 190) are required.

(2) *Sodium chloride, 0.9 percent solution.*

⁴ Howe, *Journal of Biological Chemistry*. 49, 93, and 101 (1921); Kingsley, *Journal of Biological Chemistry*, 133, 631 (1940).

(3) *Sodium sulfate, 22.5 percent solution.* Dissolve 225 gm of anhydrous Na_2SO_4 in water at about 40°C . and dilute to 1 liter. The solution should not be allowed to cool below 24°C ., as it is supersaturated at lower temperature and likely to crystallize. The maximum solubility is at 32.5°C . If crystallization occurs place the solution at 33° to 40° until resolution is complete. It may be kept permanently in an incubator at 38° .

(4) *Ether.*

c. TOTAL PLASMA NITROGEN.

(1) *Titrimetric.* Dilute 1 ml of plasma to 10 ml with 0.9 percent NaCl solution and determine the nitrogen in samples of 2 ml as described in paragraph 191.

Calculation:

$$\text{Mg total N per 100 ml plasma} = \text{Mg N in sample} \times 500.$$

(2) *Colorimetric or photometric.* Dilute 1 ml of plasma to 100 ml with 0.9 percent NaCl solution. Use samples of 1 ml for analyses as described in paragraph 192.

Calculation:

$$\text{Mg total N per 100 ml plasma} = \text{Mg N in sample} \times 10,000$$

d. ALBUMIN N PLUS NONPROTEIN N.

(1) *Precipitation and removal of globulins.*

(a) Add 1 ml of plasma to exactly 15 ml of 22.5 percent sodium sulfate solution in a 50 ml centrifuge tube. Stopper and mix thoroughly by inverting. Remove the stopper and add 6 ml of ether. Stopper again and shake vigorously for 20 to 30 seconds. This completes precipitation of the globulin. Cap the tube to avoid loss of ether and centrifuge for 8 minutes at 2,200 rpm. The precipitated globulin forms a middle layer between the clear albumin solution below and the ether above.

(b) If the room temperature is cold, the sodium sulfate may start to crystallize during the centrifuging. If crystallization occurs the analysis can be saved by warming and recentrifuging.

(c) If centrifuge equipment is not available for the above quick separation with the help of ether (Kingsley method), the mixture of plasma and sodium sulfate is incubated in a stoppered tube or 25 ml flask overnight at 38°C . to precipitate the globulins. The solution is filtered through a Whatman No. 50 filter paper in the incubator. If the first portion of the filtrate is not clear it is returned to the filter. The funnel is kept covered with a watch glass to prevent evaporation.

(2) *Determination of albumin N plus NPN in filtrate.* These two forms of nitrogen are left in the filtrate from precipitation of the globulins. The nitrogen in the filtrate is determined by microkjeldahl analysis, either titrimetric or colorimetric.

(a) *Titrimetric microkjeldahl nitrogen determination.* A sample of 3 ml of the filtrate, equivalent to 3/16 ml of plasma, is used for each microkjeldahl determination, carried out as described in paragraph 191.

Calculation:

Mg Albumin N + NPN per 100 ml of plasma = $533 \times \text{mg N in sample}$.

(b) *Colorimetric microkjeldahl nitrogen determination.* Five ml of the filtrate from the globulins is diluted to 25 ml. Of the diluted solution, 2 ml, equivalent to 1/40 ml of plasma, is used for the colorimetric analysis described in paragraph 192.

Calculation:

Mg albumin N + NPN per 100 ml of plasma = $4,000 \times \text{mg N in sample}$.

e. **NONPROTEIN NITROGEN.** Determine as described in paragraph 193.

f. **CALCULATION OF ALBUMIN AND GLOBULIN IN PLASMA.** Per 100 ml of plasma, let $a = \text{mg total N}$, $b = \text{mg albumin N} + \text{NPN}$, and $c = \text{mg NPN}$. Then calculate:

Gm globulin per 100 ml plasma = $0.00625 (a - b)$.

Gm albumin per 100 ml plasma = $0.00625 (b - c)$.

195. Fibrinogen in Plasma⁵

a. **GENERAL.** Ionized calcium in solution is necessary for coagulation of fibrin. In oxalated blood coagulation is prevented because the added oxalate precipitates the calcium of the blood. In the determination of fibrinogen the calcium of oxalated blood plasma is replaced by addition of excess calcium chloride, and coagulation of the fibrin begins at once. The nitrogen of the fibrin precipitate is determined by microkjeldahl.

b. **REAGENTS.**

- (1) *Sulfuric acid, approximately 2 N.* Dilute 5.6 ml of concentrated sulfuric acid to 100 ml with water.
- (2) *Sodium hydroxide, approximately 0.25 N.* Dissolve 1 gm of sodium hydroxide in water and dilute to 100 ml.
- (3) *Sodium chloride solution, 0.9 percent.*
- (4) *Calcium chloride solution, 2.5 percent.* Dissolve 2.5 gm of anhydrous CaCl_2 , 3.3 gm of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, or 5 gm of crystalline $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ in water and dilute to 100 ml.

⁵ Cullen and Van Slyke, *Journal of Biological Chemistry*. 41, 487 (1920), modified.

- (5) *Reagents for either the titrimetric or the colorimetric microkjeldahl analysis* (par. 191 or 192).

c. PROCEDURE.

- (1) Collect about 5 ml of blood in a flask or test tube containing 1.5 to 2.0 mg of potassium oxalate per ml of blood. Centrifuge. Pipette 1 ml of the plasma into a 50-ml beaker containing 30 ml of 0.9 percent NaCl solution and 1 ml of 2.5 percent CaCl_2 solution. Mix the plasma with the solutions by gentle swirling, and let stand at least 30 minutes, until a solid clot of fibrin forms.
- (2) Insert a glass rod into the beaker and twirl it gently, loosening the clot and winding it onto the rod. Rotate the rod gently, always in one direction against the wall of the beaker to obtain a compact clot. Do not allow the rod to touch the bottom of the beaker or the clot may be lost. Finally withdraw the rod and clot from the solution, and roll the clot gently on filter paper to remove adherent solution. The nitrogen in the clot is determined by colorimetric or titrimetric microkjeldahl analysis as follows:

d. **TITRIMETRIC MICROKJELDAHL.** The fibrin clot is obtained and dried on filter paper as described above. Then the rod with the clot is inserted to the bottom of a Pyrex test tube of 25 by 200 or 15 by 150 mm size. The clot is dislodged with a little water and the rod is withdrawn, adherent bits of clot being washed back into the bottom of the tube. The entire clot is then subjected to nitrogen determination as described in paragraph 191.

Calculation:

Gm fibrinogen per 100 ml plasma = $0.625 \times \text{mg in Kjeldahl sample}$.

e. **COLORIMETRIC MICROKJELDAHL DETERMINATION OF FIBRIN.** Place the rod with the clot in a 15- by 150-mm test tube. Add 4 ml of the 0.25 N NaOH solution and place the tube in boiling water for several minutes until the clot dissolves. Add about 10 ml of water and transfer the solution to a 25 ml volumetric flask, using several washings, of about 3 ml of water each, to complete the transfer. Dilute to the 25 ml mark, mix, and take portions of 5 ml for the colorimetric microkjeldahl analysis. (See par. 192.)

Calculation:

Gm fibrinogen per 100 ml plasma = $3.125 \times N$ in Kjeldahl sample.

196. Total Protein of Spinal Fluid, Turbidimetric

(as described by Summerson, in *Directions for Klett-Summerson Photoelectric Colorimeter*, New York, 1941)

a. GENERAL. The proteins are precipitated by sulfosalicylic acid in the form of a suspension. The concentration of the suspension is estimated from the light transmittance in a colorimeter, or, preferably, a photometer.

b. REAGENTS.

(1) *Sulfosalicylic acid, 3 percent water solution.*

(2) *Stock standard protein solution.* Dilute 5 ml of normal human serum to 50 ml with 15 percent NaCl solution. Mix thoroughly and filter. Determine the total nitrogen of the diluted filtrate by microkjeldahl analysis. (The nitrogen content of the filtrate is of the order of 1 mg per ml. Hence 2 ml may be used for a titrimetric microkjeldahl (par. 191), or 0.2 ml for a colorimetric (par. 192.) Calculate the mg of nitrogen in 100 ml of filtrate as

$$\frac{\text{mg of N in sample} \times 100}{\text{ml of filtrate used as sample}}$$
. From the total mg N per

100 ml of filtrate thus found, subtract 3 mg for the non-protein nitrogen, and multiply the difference by 6.25 to obtain the mg of protein per 100 ml of filtrate, which will be about 700 mg. This standard keeps well if stored in a refrigerator.

(3) *Dilute working standard protein solutions.* Working standards are prepared by diluting the stock standard with water. A generally useful standard is one prepared by twentyfold dilution of the stock, so that the protein content is about 35 mg per 100 ml.

c. PROCEDURE.

(1) Place 1 or 2 ml of the clear, colorless spinal fluid in a colorimeter cup or a photometer cuvette and add 4 volumes (4 or 8 ml) of the 3 percent sulfosalicylic acid solution. Mix gently, preferably by inversion. The reading may be made at any time between 10 and 30 minutes after addition of the sulfosalicylic acid. Treat 1 or 2 ml of the working standard in the same way at approximately the same time.

(2) Just before the reading, gently mix both suspensions again.

(3) If the protein content of the unknown is obviously much lower than that of the working standard, prepare a more dilute standard.

(4) If the protein in the spinal fluid flocculates after addition of the sulfosalicylic acid, dilute another portion of the original spinal fluid to 2 or 4 volumes, giving $d = 2$

or 4 in the calculation (below), and precipitate a portion of the diluted fluid.

d. MEASUREMENT IN COLORIMETER. Unknown and standard are compared, preferably with a blue light filter.

Calculation:

$$\text{Mg protein per 100 ml spinal fluid} = C_s \times d \times \frac{S}{U}.$$

C_s is the mg protein per 100 ml in the working standard used, d is the number of volumes to which the spinal fluid was diluted before a portion was taken for analysis. S is the reading of the standard, U the reading of the unknown.

e. MEASUREMENT IN PHOTOMETER. Light of about 450 millimicrons wave length is used. The zero is set with a water blank.

Calculation:

$$\text{Mg protein per 100 ml spinal fluid} = C_s \times d \times \frac{D_u}{D_s}.$$

C_s and d have the same significance as above, D_u is the optical density of the precipitated spinal fluid, D_s the density of the standard.

f. QUALITATIVE TEST FOR INCREASED SPINAL FLUID PROTEIN — PANDY'S TEST. This is a qualitative test to reveal an *abnormal* increase in spinal fluid protein. The reagent, a saturated solution of phenol in water, is prepared as follows: Place a beaker containing crystalline phenol, CP, in a water bath and raise the temperature until melting occurs (45° to 55° C.). Pour 100 ml of the melted phenol into a suitable container and add sufficient distilled water to make 1,000 ml volume. Shake at intervals during several days in which it is kept in an incubator. Use the clear supernatant fluid. The test is performed by adding 1 large drop of spinal fluid to 1 ml of the reagent. The immediate formation of a bluish-white cloud around the drop as it is swirled gently in the reagent indicates a definite increase in protein. It should not be confused with a faint trace. Results of the test are reported as negative, moderate increase, or marked increase. To insure uniformity of results it is recommended that the phenol solution be kept in an incubator.

197. Urea of Blood or Plasma, Titrimetric⁶

a. GENERAL. The principle, reagents, and apparatus are the same described in paragraphs 172 and 173 for determining urea in urine. The determination in blood is simpler than in urine,

⁶Van Slyke and Cullen, *Journal of Biological Chemistry*, 19, 211 (1914), and 24, 117 (1916), modified by titration of the ammonia in boric acid solution.

however, because the ammonia content of the blood is negligible and need not be determined as part of the analysis. Hence, in place of the four aeration tubes for each analysis required with urine, only two are required for each blood analysis, one tube for the blood and one for the boric acid. The procedure is convenient where many blood urea determinations are required, because it is easy to run numbers of aerations at one time.

b. PROCEDURE.

- (1) A sample of 3 ml of blood is mixed with 3 ml of phosphate buffer solution in an aeration tube (fig. 16 in par. 172). Five drops of caprylic alcohol are added, and 0.5 ml of the 10 percent urease solution, which is at once well mixed with the blood. The stopper bearing the aeration inlet and outlet tubes is set in place, and digestion with the urease is continued only for the length of time found necessary. (See par. 172 *e* (5)). Unnecessarily prolonged contact between blood and enzyme is to be avoided, because of the possibility of slow formation of ammonia by action of arginase in the blood cells on canavanine that may be present in the urease preparation. Ten minutes usually suffices.
- (2) The aeration is carried out as in the urine analysis. Rarely a blood will foam despite the 5 drops of caprylic alcohol. The foaming may begin only after the aeration has proceeded for some time. Further addition of caprylic alcohol does not stop it. Two or three ml of ethyl alcohol are introduced by momentarily disconnecting the rubber tubing from the inlet of the blood tube and admitting alcohol through the inlet tube, while the aeration proceeds at a slow rate.

c. CALCULATION. $Mg\ urea\ N\ per\ 100\ ml\ blood = 5\ (A - B)$. *A* is the ml of 0.01071 *N* sulfuric acid used in the titration of ammonia from the blood tube; *B* is the ml of 0.01071 *N* sulfuric acid used in titrating the blank on the reagents.

198. Urea of Blood or Plasma, Colorimetric⁷

a. GENERAL. The urea is hydrolyzed to ammonia by the action of urease, the blood proteins are precipitated, and the ammonia in the filtrate is determined by Nesslerizing and measuring the optical density of the solution in a colorimeter or photometer. Constitutents of the blood filtrate other than ammonia have a

⁷ Gentzkow and Masen, *J. Biol. Chem.*, 143, 531 (1942).

negligible effect on the light transmittance by the Nesslerized solution, if light of wave length 500 millimicrons is used.

b. REAGENTS.

- (1) *Sodium tungstate, 10 percent.* Ten gm $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ per 100 ml.
- (2) *Sulfuric acid, 0.67 normal.* Dilute 67 ml of 1 N sulfuric acid to 100 ml.
- (3) *Ammonium sulfate, standard solution with 0.015 mg of nitrogen per ml.* Of the stock solution with 0.1 mg of nitrogen per ml (par. 192 b (4)) dilute 15 ml to 100 ml with 0.01 N sulfuric acid.
- (4) *Potassium gluconate solution, 1 percent, aqueous.* Store in refrigerator. Make up fresh weekly.
- (5) *Potassium persulfate, 2.5 percent solution.* Use the special nitrogen-free persulfate described for use in colorimetric microkjeldahl analyses. (see par. 192.)
- (6) *Urease solution.* This contains 20 mg of Squibbs' "double strength" urease per ml.
- (7) *Nessler's solution.* See paragraph 192.

c. APPARATUS.

- (1) Volumetric flasks of 50 ml capacity.
- (2) Test tubes calibrated to hold 20 ml and provided with parafined stoppers.
- (3) If a Duboscq type colorimeter is used, a No. 75 Wratten gelatin filter, or other filter transmitting light chiefly of wave length 500, is cemented between cover slips and used in the eyepiece of the colorimeter. Or it is cemented between microscope slides and used over the source of light in the colorimeter base. Sand-blasting one surface of the slides improves diffusion. If the usual light comes through a blue "daylight" filter, remove the filter. The comparison of the Nessler color of the standard with that of the blood filtrate is rendered inaccurate by unidentified constituents of the filtrate unless the light has a wave length of 500 millimicrons.

d. PROCEDURE.

- (1) *Digestion and filtration.*
 - (a) Pipette 5 ml of whole oxalated blood into a 50 ml volumetric flask. Add enough water to fill the flask half full, then 1 ml of the urease solution. Mix by whirling and let stand at room temperature long enough for the enzyme to act (par. e below for time required) but no longer, as unnecessarily long digestion may lead to formation of slight amounts of urea from the action of cellular arginase on canavanine in the urease prep-

aration. When digestion is complete add 5 ml of the tungstate solution, mix, add 5 ml of the 0.67 *N* sulfuric acid, and water nearly to the 50 ml mark. Mix thoroughly and let stand 10 minutes. Fill to the mark and mix. (See *b* below.) Filter through a good grade of qualitative filter paper. Whatman No. 2 is satisfactory. Do not use acid washed filter papers, as they give appreciable ammonia blanks. After filtration has proceeded about 10 minutes pour the filtrate back onto the paper. The first portion of the filtrate gives too high values for urea N and NPN.

- (b) If the blood urea nitrogen is over 100 mg per 100 ml the 5 ml of 0.67 *N* sulfuric acid will not suffice to make the precipitation of the proteins complete, because about 0.5 ml of the acid is neutralized by the ammonia formed from blood urea in the concentration of 100 mg of nitrogen per 100 ml. With such a blood, the supernatant solution over the precipitate will be cloudy. Add another 0.5 or 1.0 ml of the 0.67 *N* H₂SO₄ and mix before the mixture is made up to 50 ml and filtered.

(2) *Nesslerization.*

- (a) Place 5 ml of the filtrate in a test tube graduated at 20 ml. In a similar tube place 5 ml of the standard solution, containing a total of 0.075 mg of ammonia *N*. To each tube add water to the 20 ml, mark and mix.
 - (b) Prepare a Nesslerizing solution by mixing 5 ml of gluconate solution, 5 ml of the persulfate solution, and 10 ml of Nessler's solution. The mixture must be used within the next 15 minutes. The persulfate and gluconate prevent development of turbidity when the Nessler's solution is added to the blood filtrate; without these reagents some reducing material in the filtrate might reduce mercury in the Nessler's solution and cause turbidity.
 - (c) To the standard and to the unknown add 5 ml portions of the Nesslerizing solution, measured accurately with a transfer pipette or a burette. Insert paraffined stoppers into the tubes and mix the solutions by repeated inversion. Allow to stand 15 minutes for the color to develop. Within the next hour compare the standard and the unknowns.
- (3) *Colorimetric measurement.* With the No. 75 Wratten filter in place on the colorimeter, the comparison of the

unknown and the standard is made in the usual manner. When 5 ml of blood filtrate is Nesslerized the calculation is:

$$\text{Mg urea N per 100 ml blood} = 15 \times \frac{S}{U}$$

S is the reading of the standard, U that of the unknown.

If the ratio, $S:U$, is greater than 2 (blood urea N over 30 mg per 100 ml), repeat the Nesslerization of the filtrate, using, instead of 5 ml of filtrate, 3, 2, or 1 ml. The calculation then becomes:

$$\text{Mg urea N per 100 ml blood} = \frac{75}{V} \times \frac{S}{U}$$

V is the ml of filtrate used.

(4) *Photometric measurement.*

- (a) A portion of filtrate is Nesslerized as described above, and the ammonia content is determined with light of wave length 500 millimicrons. Gentzkow and Masen found that the nonurea constituents of the blood filtrate affect the results if wave lengths above or below 500 are employed.
- (b) The standard is prepared in the same way as for colorimetric measurement.
- (c) A blank solution is prepared with all the reagents, but with water in place of the 5 ml of blood. When 5 ml of blood filtrate is Nesslerized, the calculation is:

$$\text{Mg urea N per 100 ml blood} = 15 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown, D_s the density of the standard.

If a volume, V ml, of filtrate is used, the calculation is:

$$\text{Mg urea N per 100 ml blood} = \frac{75}{V} \times \frac{D_u}{D_s}$$

e. DETERMINING TIME REQUIRED FOR DIGESTION BY UREASE.

- (1) Prepare a solution of urea containing 300 mg per 100 ml. Of a blood of previously determined urea content, pipette portions of 5 ml into each of four 50-ml volumetric flasks. To each flask add also 5 ml of the 0.3 percent urea solution. Then proceed with determination of the urea as above outlined, except that the duration of the digestion is varied. After the enzyme

has acted for intervals of 10, 20, 30, and 40 minutes in the four respective flasks, its action is stopped by addition of tungstate and sulfuric acid. In order to neutralize the ammonia formed by the large amount of urea, add 5.5 ml of the 0.67 *N* sulfuric acid instead of the usual 5 ml. The ammonia formed is measured in the filtrates as described for blood analyses. The interval is thus ascertained that is necessary, at the temperature employed, to liberate the maximal amount of ammonia. The amount of urea added should raise the determined urea nitrogen content of the blood by 140 mg per 100 ml.

- (2) If the urease is later used at temperatures other than that at which the digestion time was determined, it may be necessary to make allowances for the effect of the temperature on the speed of enzyme action. A fall of 5° makes the enzyme take 1.4 times as long; a fall of 10° approximately doubles the time required.
- (3) It is essential to test the activity of the urease as described above before beginning to use it. A preparation that has full activity when prepared may deteriorate during transportation or storage, especially if permitted to absorb moisture. Keep the urease powder in a tightly stoppered bottle; if the atmosphere is humid it is well after the bottle has once been opened, to keep it in a desiccator.
- (4) Urease of good activity will complete digestion under the above conditions in 20 minutes at 20°. If the enzyme takes twice as long, however, the time can be reduced to 20 minutes by using twice as much urease.
- (5) Use only Squibb's "double strength" powdered urease. Other preparations may contain substances that interfere with the colorimetric analysis.

199. Urea Clearance

a. DIRECT COLORIMETRIC COMPARISON OF BLOOD AND URINE.⁸

(1) *General.*

- (a) The blood urea clearance is the volume of blood cleared of urea per minute by renal excretion. A normal man with abundant flow of urine excretes per minute on the average the amount of urea in 75 ml of his blood, and his clearance is said to be 75. When the kidneys

⁸ Van Slyke and Cope, *Proceedings of the Society of Experimental Biology and Medicine*, 129, 1169 (1932) modified by use of the Nesslerizing technic of Gentzkow and Masen. (See par. 173.)

suffer decrease of excretory ability the urea clearance decreases proportionally. When the clearance falls *permanently* to the neighborhood of 4 ml per minute, or 5 percent of normal, death in uremia is likely to occur regardless of the nature of the renal changes that cause the decrease. Temporary fall in the clearance, without renal pathological condition, may occur when shock or other transitory condition decreases the volume of blood flowing through the kidneys.

- (b) When the clearance is 75, if the urine excreted in a minute is diluted to 75 ml, the concentration of urea in the diluted urine will be the same as the concentration of urea in the blood. The ratio, (urea concentration in diluted urine): (urea concentration in blood), will then be 1, and the percentage of average normal clearance will be 100. Measurement of the urea clearance can thus be made by diluting the urine passed in one minute to 75 ml, and measuring the ratio, (urea concentration in diluted urine): (urea concentration in blood). This ratio times 100 gives the percentage of normal kidney function in terms of the urea clearance.
- (c) The ratio can be directly observed in a Duboscq colorimeter as the ratio, $B : U$, B being the reading of the cup with Nesslerized blood filtrate and U the reading of the cup with the Nesslerized, diluted urine, both fluids being treated with urease, as in colorimetric urea determination. If the comparison is made in a photometer, the ratio used is $D_u : D_b$ in which D_u and D_b are the optical densities of the Nesslerized solutions prepared from diluted urine and from blood filtrate, respectively. In the colorimeter the ratio of the readings varies inversely as the ratio of the urea concentrations, while in the photometer the optical densities vary directly as the concentrations; hence the ratio of readings used is $B : U$ for the colorimeter, but $D_u : D_b$ for the photometer. Since the urea concentrations in urine and blood are compared directly, the actual concentrations do not need to be determined, and no standard solutions are necessary.
- (d) In table XVI are given the dilution factors which express the number of times that urine excreted at different rates must be diluted to make its urea concentration equal to that of the blood when the clearance is 100 percent of mean normal. When the

rate of urine excretion in an adult exceeds 1.93 ml per minute the dilution factor is calculated as $75/V$, V being the urine volume in ml excreted per minute. When V is less than 1.93 (an average value) the normal clearance decreases as the square root of V . Correction for this retarding effect of low urine volumes is made by calculating the dilution factor as

$$\frac{75}{V} \times \sqrt{\frac{V}{1.93}} = \frac{54}{\sqrt{V}}, \text{ when } V \text{ is less than } 1.93. \text{ A child}$$

has a smaller clearance than an adult, the clearance decreasing as a function of the child's height. Hence when the clearance of a child is determined a correction is made by multiplying the observed V by a correction factor from the last column of the table, and estimating the number of times to dilute the urine from the corrected V .

(2) *Reagents.*

- (a) *Those for colorimetric determination of blood urea.* (See pars. 172, 173, and 197.)
- (b) *Acetate buffer.* Dissolve 15 gm of crystalline sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) in distilled water in a 100 ml volumetric flask, add 1 ml of glacial acetic acid, and dilute to 100 ml with water.
- (c) *Permutit powder* for absorbing ammonia from solutions.

(3) *Apparatus.*

- (a) *Measuring cylinders* (50, 100, and 250 ml).
- (b) *Volumetric flasks* (50 ml).
- (c) *Duboscq colorimeter or a photometer.*

(4) *Procedure.*

(a) *Handling patient.*

1. No preliminary preparation of the patient is necessary, except that vigorous exercise should be avoided immediately before the test. Separate collections are made of the urine secreted into the bladder during two successive collection periods, which are each conveniently, but not necessarily, about 1 hour. The two urine specimens are collected in order to obtain duplicate values for the clearance. The chief sources of error are inaccurate timing of the collection periods, and incomplete voiding of the bladder, rather than errors in the analytical parts of the clearance procedure. Hence it is de-

sirable to have results from two separate collection periods, to serve as checks on each other.

2. At the beginning of the 2-hour double period, and again at the end of the first hour, the subject is given a glass of water. The subject urinates at the beginning of the test, and the time to the nearest minute is registered at the moment he finishes voiding. At the end of about 1 hour the patient voids again, and the time, accurate to 1 minute, is recorded when he completes this voiding. The time between these two voidings constitutes the *first excretion period*.
 3. After the second voiding the subject receives a second glass of water, and a little over 5 ml of blood is drawn and oxalated for analysis.
 4. About 1 hour later the subject voids a third time. The exact time at the end of this voiding is recorded. The interval between the second and third voidings constitutes the *second excretion period*.
 5. Use of excretion periods of 1 hour is not essential. It is essential only that the *time of each period be accurately measured* so that the urine output per minute can be calculated. Excretion periods of several hours can be used if circumstances render it desirable. With infants a collection bottle is attached over the urinary outlet, and the flow is collected over whatever is the voluntary excretion period.
 6. The volume of urine collected during each period is carefully measured in a cylinder. If the volume is less than 50 ml measure it in a 50 ml cylinder, if between 50 and 100 ml use a 100 ml cylinder, etc., employing a cylinder only large enough for the measurement. If the volume for 1 hour's period is below 25 ml discard this specimen, since collections are not reliable if the urine flow is too slow.
- (b) *Treatment of blood.* A 5 ml sample of blood is treated with urease, precipitated, and Nesslerized, exactly as described for the colorimetric determination of blood urea. (See par. 198.)
- (c) *Treatment of urine.*
1. Of the urine collected in each period, about 10 ml is placed in a 50 ml Erlenmeyer flask, 2 gm of permutit powder is added, and the mixture is rotated for 5

Table XVI. Number of Times to Dilute Urine for Urea Clearance

Number of times to dilute urine of adults						Correction factors for children under 15	
V Volume of urine per minute*	Number of times to dilute urine	V Volume of urine per minute*	Number of times to dilute urine	V Volume of urine per minute*	Number of times to dilute urine	Height	Factor to multiply V to obtain corrected V
ml		ml		ml		cm†	
0.50	76	1.25	48.3	3.50	21.4	175	1.01
0.52	75	1.30	47.4	3.60	20.8	170	1.06
0.54	73	1.35	46.5	3.70	20.3	165	1.10
0.56	72	1.40	45.6	3.80	19.7	160	1.17
0.58	71	1.45	44.8	3.90	19.2	155	1.25
0.60	70	1.50	44.1	4.00	18.7	150	1.33
0.62	69	1.55	43.4	4.10	18.3	145	1.41
0.64	68	1.60	42.7	4.20	17.9	140	1.49
0.66	67	1.65	42.1	4.30	17.4	135	1.59
0.68	66	1.70	41.4	4.40	17.0	130	1.69
0.70	65	1.75	40.8	4.50	16.7	125	1.80
0.72	64	1.80	40.3	4.60	16.3	120	1.92
0.74	63	1.85	39.7	4.70	16.0	115	2.04
0.76	62	1.90	39.2	4.80	15.6	110	2.19
0.78	61	1.95	38.5	4.90	15.3	105	2.34
0.80	60	2.00	37.5	5.00	15.0	100	2.50
0.82	60	2.10	35.7	5.20	14.4	98	2.56
0.84	59	2.20	34.1	5.40	13.9	96	2.62
0.86	58	2.30	32.6	5.60	13.4	94	2.68
0.88	58	2.40	31.2	5.80	12.9	92	2.74
0.90	57	2.50	30.0	6.00	12.5	90	2.81
0.92	56	2.60	28.8	6.20	12.1	88	2.90
0.94	56	2.70	27.8	6.40	11.7	86	2.99
0.96	55	2.80	26.8	6.60	11.4	84	3.09
0.98	55	2.90	25.8	6.80	11.0	82	3.20
1.00	54	3.00	25.0	7.00	10.7	80	3.31
1.05	53	3.10	24.2	7.20	10.4	78	3.42
1.10	51	3.20	23.4	7.40	10.1	76	3.56
1.15	50	3.30	22.7	7.60	9.9	74	3.70
1.20	49.3	3.40	22.0	7.80	9.6	72	3.86

*For a child use corrected V. To obtain this, multiply observed V by the correction factor (last column) that corresponds to the height.

†Cm = inches $\times$ 2.54.

minutes to permit the permutit to absorb the ammonia.

2. If the patient is an *adult*, 1 ml of the permutit-treated urine from each of the two periods is diluted in a measuring cylinder as indicated by table XVI, the degree of dilution depending on the volume of urine voided per minute.
3. If the patient is a *child*, a correction is made for body size by multiplying the observed ml of urine per

minute by a factor, in the last column of the table, depending on the height of the child. The product is the *corrected urine flow* per minute. The corrected figure is used to estimate the number of times the permutit-treated urine is to be diluted. For example, if the patient is a child 140 cm tall, the correction factor in the last column is 1.49. If the observed urine flow is 1.70, the corrected flow is $1.70 \times 1.49 = 2.53$ ml per minute, and 1 ml of the permutit-treated urine is diluted to 29.5 ml.

4. After proper dilution of samples of the two urines, place 5 ml of each diluted specimen in a 50 ml volumetric flask. Fill the flask about half full with water, and add 1 ml of the acetate buffer and 1 ml of the 2 percent urease solution. Mix the liquids, and let the mixture stand long enough for the urease to complete its action. (See par. 198.) Then add 2.5 ml of 10 percent of sodium tungstate, 2.5 ml of the 0.67 *N* sulfuric acid, or 3.0 ml if the blood urea is over 100 mg, dilute to 50 ml, and filter, as described for the colorimetric determination of urea in blood. (See par. 198.) Of the filtrate, 5 ml is Nesslerized, with dilution to 25 ml, as described for the colorimetric determination of blood urea. (See par. 198.)
- (d) *Colorimetric comparison of blood and urine filtrates.*
 1. The Nesslerized blood filtrate is placed in the left-hand cup of the colorimeter, and is compared in succession with the Nesslerized filtrates of the two urine specimens, which are placed successively in the right cup. The comparison is preferably made with a No. 75 Wratten filter in the eyepiece.
 2. If the ratio of the reading in the blood cup to the reading in the urine cup is over 0.50 (clearance over 50 percent of average normal), the two solutions are close enough to make the reading accurate, and it is accepted. If, however, the ratio is below 0.50, another portion of blood filtrate (3, 2, or 1 ml) is Nesslerized and is compared with the urine filtrates.

Calculation: When 5 ml of blood filtrate is used the calculation is:

$$\text{Urea clearance (percentage of average normal)} = \frac{100 B}{U}.$$

B is the reading of the colorimeter cup with the blood filtrate, and *U* is the reading of the cup with the urine filtrate.

When less than 5 ml of blood filtrate is used the calculation is:

$$\text{Urea clearance (percentage of average normal)} = 20 F \times \frac{B}{U}$$

F is the number of ml of blood filtrate taken for Nesslerization.

(e) *Photometric comparison of blood and urine filtrates.*

The optical densities of the blood and urine filtrates may be measured in a photometer, with wave length 500 millimicrons. The calculation then is:

$$\text{Urea clearance (percentage of average normal)} = 20F \times \frac{D_{\text{urine}}}{D_{\text{blood}}}$$

F as above, is the ml of blood filtrate taken for Nesslerization, D_{urine} is the optical density of the urine filtrate, and D_{blood} the optical density of the blood filtrate, Nesslerized.

b. **ALTERNATIVE CALCULATION OF UREA CLEARANCE FROM SEPARATE ANALYSES OF BLOOD AND URINE.** From the volume flow of urine per minute and the urea concentrations of blood and urine, determined by either the colorimetric or the aeration-titration method, the clearances can be calculated:

- (1) Indicating by V the ml of urine excreted per minute, when V is over 1.93 the calculation is:

$$\text{Urea clearance (percent of average normal)} = 1.33V \times \frac{\text{mg urea N per 100 ml urine}}{\text{mg urea N per 100 ml blood}}$$

- (2) When V is under 1.93 the calculation is:

$$\text{Urea clearance (percent of average normal)} = 1.85\sqrt{V} \times \frac{\text{mg urea N per 100 ml urine}}{\text{mg urea N per 100 ml blood}}$$

Example: $V = 1.44$ ml per minute; urine urea nitrogen = 765 mg per 100 ml; blood urea N = 14.6 mg per 100 ml.

$$(3) \text{ Clearance} = 1.85 \times 1.20 \times \frac{765}{14.6} = 116 \text{ percent of average normal, } 1.20 \text{ being the square root of } 1.44.$$

200. Uric Acid in Plasma or Serum⁹

a. **GENERAL.** The ability of uric acid to reduce the hexavalent tungsten of phosphotungstic acid to lower valence, with formation of a blue color, first utilized by Folin, is employed in this method. The present application of the reaction has the advantages that the reagents are easily prepared, stable, and nonpoisonous, and that the reaction occurs at room temperature. Clouding, which

⁹ Kern and Stransky, *Biochem. Z.*, 190, 419 (1937), modified by Archibald.

has been a threat in phosphotungstic acid reduction methods for uric acid, is prevented by a mixture of glycerol, sodium silicate, and polyanethol sulfonate (Liquoid La Roche). The reduction belongs to the class of incompletely defined oxidation-reduction reactions (sec. VI, ch. 3), and accuracy depends on precise observance of empirically fixed conditions.

b. REAGENTS.

- (1) *Stock standard solution of uric acid, 1 mg per ml.* Exactly 1 gm of uric acid is placed in a 250 ml Erlenmeyer flask. Approximately 0.5 gm of lithium carbonate is dissolved in 150 ml of hot water, the hot solution is added to the uric acid, and the mixture is stirred until all the uric acid is dissolved. Transfer the solution to a liter volumetric flask, and follow it with two portions of 150 ml of water each, which are used to rinse the Erlenmeyer flask. Add 25 ml of 40 percent formaldehyde and mix. Add 3 ml of glacial acetic acid, mix, and shake to remove CO₂. When the evolution of CO₂ from the lithium carbonate has stopped make up to 1 liter and mix. Store in a brown bottle in a dark place. The solution should be renewed once a year.
- (2) *Dilute working standard of uric acid, 0.005 mg per ml.* Dilute 1 ml of the above stock standard to 200ml with water. The working standard solution is made fresh on the day it is to be used.
- (3) *Polyanethol sodium sulfonate* (Liquoid La Roche).¹⁰ Dissolve 1 gm in 50 ml of water. Store in an ice box.
- (4) *Glycerol-silicate reagent.* Dissolve 10 gm of Merck's crystalline sodium silicate "soluble" in 100 ml of hot water, mix with 20 ml of glycerol, and cool. If the solution is even slightly cloudy filter through hardened filter paper.
- (5) *Phosphotungstic acid solution.* To 50 gm of A.C.S. grade sodium tungstate (Na₂WO₄·2H₂O), add 400 ml of water and 40 ml of concentrated (85 percent) phosphoric acid. Boil under a reflux condenser gently for 2 hours. Make up to 500 ml and store in a brown bottle. (Commercial phosphotungstic acid should not be substituted for this solution.)
- (6) *Sodium hydroxide solution, 0.5 N.*

c. PROCEDURE.

- (1) *Precipitation of proteins.* Pipette 2 ml of plasma or serum into a 50 ml Erlenmeyer flask and add 16 ml of

¹⁰ Liquoid La Roche can be obtained on Army order from Dr. H. M. Wuest, Director of Research, Hoffman La Roche, Inc., Nutley, New Jersey.

water and 1 ml of the 0.5 normal NaOH. Then add 1 ml of the phosphotungstic acid solution, making the total volume 20 ml. The first half of the phosphotungstic acid may be added rapidly, but the last half should be added slowly, drop by drop, with constant whirling of the solution. Precipitation of the proteins is completed by the last drops of the phosphotungstic acid. The mixture is let stand 5 or more minutes, and is then filtered through a dry paper of 8 or 9 cm diameter. (Half the amounts of plasma and reagents can be used if desirable.)

- (2) *Development of color in plasma filtrate.* Transfer 10 ml of the filtrate to a 25 ml flask or test tube. Add 5 ml of the glycosilicate reagent, mix; then add 1 ml of the polyanethol sulfonate solution and mix again. Finally add 1 ml of the phosphotungstic acid solution, making a total volume of 17 ml, and mix. Allow to stand 15 minutes for the color to develop. The color then remains practically constant for ½ hour or more, then begins to fade.
- (3) *Standard.* Treat 10 ml of the dilute working standard the same way at the same time as the plasma filtrate.
- (4) *Photometric blank* (not required for Duboscq colorimeter). Treat 10 ml of water in the same way as the plasma filtrate.
- (5) *Colorimetric measurement.* The unknown and standard are compared.

Calculation:

$$\text{Mg uric acid per 100 ml plasma} = 5 \times \frac{S}{U}$$

S is the reading of the standard, *U* is the unknown.

- (6) *Photometric measurement.* Light of wave length 700 millimicrons is used. The zero point of optical density is set with the photometric blank. Readings are taken on the plasma filtrate and on the standard.

Calculation:

$$\text{Mg uric acid per 100 ml plasma} = 5 \times \frac{D_u}{D_s}$$

D_u is the density of the unknown, *D_s* of the standard.

201. Creatinine in Plasma or Whole Blood¹¹

a. GENERAL. Creatinine reacts with alkaline picrate to form an intense red color, the Jaffe reaction. The color reaction is not

¹¹Folin-Wu, *Journal of Biological Chemistry*, 38, 87 (1919), modified by Phillips.

specific for creatinine; it is given by other substances in blood. However, the chromogenic material measured in normal plasma by this reaction is, according to the best evidence, 90 to 95 percent creatinine. A large part of the chromogenic material in the cells is not creatinine. Consequently the analysis is preferably made on plasma. The alkaline picrate itself has color, which decreased the accuracy of the creatinine readings in the original Folin-Wu method. In Phillips' modification, to diminish this error, 1/5 as much picrate is used, and the color is given 50 instead of 10 minutes to develop.

b. REAGENTS.

- (1) *Ten percent sodium tungstate and N/12 sulfuric acid*, described in paragraph 190.
- (2) *One percent picric acid solution*. "Reagent" grade picric acid is used. It usually contains 10 to 20 percent of moisture. Twelve gm are made up to 1 liter and the picric acid concentration is checked by titrating a 20 ml portion with 0.1 N NaOH, using phenolphthalein indicator. Of the 0.1 N NaOH 8.72 ml should be required. Adjust the concentration of the picric acid until the titration is 8.72 ± 0.1 ml. (See par. 132.)
- (3) *Dilute alkaline picrate solution*. Twenty ml of the 1 percent picric acid and 10 ml of 10 percent NaOH solution are mixed and diluted with water to 100 ml. Since the solution deepens in color for some hours or days it is desirable, though not necessary, to prepare it one or more days before using in order to make its color effect constant during a series of analyses. The solution is usable for about 1 month.
- (4) *Stock standard of creatinine solution, 0.1 percent*. One gm of creatinine, or 1.602 gm of creatinine zinc chloride, is dissolved and made up to 1 liter of 0.1 N HCl.
- (5) *Dilute standard creatinine solution*. Measure 6.0 ml of the stock 0.1 percent creatinine solution into a 1-liter volumetric flask, add 10 ml of 0.1 N HCl, and dilute to the mark with water. One ml contains 0.006 mg of creatinine.

c. PROCEDURE FOR COLORIMETRIC MEASUREMENT.

- (1) Prepare a 1:10 tungstic acid filtrate of blood, or, preferably, plasma as described in paragraph 190. Place 10 ml of the filtrate in a small flask or a test tube. In four other tubes or flasks place 5, 10, 15, and 20 ml of the dilute standard creatinine solution, adding enough water from a burette to each of the first three to make the

volume up to 20 ml. To the 10 ml of tungstic acid filtrate add 5 ml of the alkaline picrate solution, and to each 20 ml standard solution add 10 ml of the alkaline picrate solution. The blood filtrate is thus diluted to 15 ml, and each standard solution is diluted to 30 ml. The additions are all made as nearly as possible at the same time. The solutions are allowed to stand 50 minutes for the Jaffe color to develop.

- (2) The filtrate is matched against the standard to which it is nearest in color. It is desirable to use a light filter with maximal transmission at about 520 millimicrons. Such a filter minimizes the interference by the color of the picrate; without a filter the standard and unknown must be nearly alike, in order to prevent appreciable error from the color of the picrate.

- (3) *Calculation:*

$$\text{Mg creatinine per 100 ml plasma or blood} = \frac{0.3 C S}{U}$$

S and U are the readings of the standard and unknown; C is the ml of dilute standard creatinine solution measured into the final standard solution against which the plasma filtrate is matched.

d. PROCEDURE FOR PHOTOMETRIC MEASUREMENT.

- (1) The analysis with a photometer is both more accurate and more convenient than with a colorimeter. In the photometric analysis use of a blank with the same concentration of picrate as that in the unknown entirely prevents error from the picrate. The colors in both the filtrate and the standards are developed as described above. The blank reading for zero optical density is taken with a mixture of 1 volume of alkaline picrate solution and 2 volumes of water. A wave length of 520 millimicrons is used. Temperature changes affect markedly the optical density of the Jaffe reaction product, about 2 percent for each degree C. Hence the standard curve must be made, or the k constant of the calculation formula determined, at the same temperature at the analysis. The blank reading on the alkaline picrate must also be taken at the same temperature.
- (2) The optical density curve is linear: hence it may be plotted, or the k value of the calculation formula (pars. 151 and 157) determined from a single standard. A "final" standard solution equivalent to 6 mg of creatinine

per 100 ml of plasma, prepared with 20 ml of the dilute standard creatinine solution as described for the colorimetric procedure, may be used.

- (3) *Calculation.* If maximal accuracy is desired, a standard as described in (2) above is prepared with each series of analysis, and its optical density used as the basis of calculation:

$$\text{Mg creatinine per 100 ml of blood or plasma} = 6 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown, D_s the density of the standard equivalent to 6 mg percent blood creatinine. This procedure automatically corrects for temperature, and for any variation in the setting of the wave length, which as in readings of Nesslerized solutions (par. 192), is important.

- (4) For approximate routine analyses one may determine k as $6/D_s$ and use it for subsequent analyses. Then:

$$\text{Mg creatinine per 100 ml blood or plasma} = k D_u$$

202. Sugar of Blood, Plasma, or Spinal Fluid¹²

a. GENERAL. The sugar when heated with alkaline cupric tartrate solution reduces the divalent copper to cuprous oxide (Cu_2O). The cuprous oxide precipitate is made to react with a phosphomolybdate solution, in which it reduces the hexavalent molybdenum to a form of lower valence, which forms an intense blue color. Under specified conditions the amounts of Cu_2O and of colored molybdate complex are proportional to the amount of sugar. However, the reductions of both Cu^{++} and Mo^{+++++} belong to the class of incompletely defined oxidation-reduction reactions, discussed in section VI, chapter 3, and consistent results depend on exact adherence to empirically set conditions. Blood filtrates contain reducing substances other than glucose which reduce cupric tartrate; of the 100 mg. of "sugar" per 100 ml of blood determined by this method in average normal blood, about 20 mg is nonglucose reducing substances.

b. REAGENTS.

- (1) *Stock glucose solution, 1 percent.* One gm of crystalline anhydrous dextrose and 0.25 gm of benzoic acid are dissolved in water and diluted to 100 ml. The benzoic acid prevents decomposition of the sugar.
- (2) *Working standard solutions of glucose, 0.1 and 0.2 mg per ml.* The solution with 0.1 mg per ML is prepared from

¹² Folin and Wu, *Journal Biological Chemistry*, 38, 106 (1919).

the stock solution by diluting 5 ml of the latter to 500 ml with 0.25 percent benzoic acid solution. The working standard with 0.2 mg per ml is prepared by diluting 5 ml of the stock to 250 ml with 0.25 percent benzoic acid solution.

- (3) *Alkaline copper tartrate solution, colorimetric.* Dissolve 40 gm of pure anhydrous Na_2CO_3 in about 400 ml of distilled water in a liter volumetric flask. Add 7.5 gm of tartaric acid, stir until dissolved, then add 4.5 gm of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Stir until dissolved, dilute to 1 liter, and mix by inversion.
- (4) *Alkaline copper tartrate solution, photometric.* To 200 ml of the "colorimetric" alkaline tartrate solution add 100 ml of water, and mix.
- (5) *Phosphomolybdate solution.* Place 35 gm of molybdic acid anhydride (MoO_3 , commonly sold as "molybdic acid") and 5 gm of sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) in a liter beaker. Add 200 ml of 10 percent sodium hydroxide solution and 200 ml of distilled water. Boil vigorously for 30 minutes. Cool, wash into 500 ml volumetric flask, using enough water to bring the volume of solution to about 350 ml. Then add 125 ml of concentrated (85 percent) phosphoric acid, mix, add water to the 500-ml mark, and mix again.

c. PROCEDURE FOR COLORIMETRIC MEASUREMENT.

- (1) *Reduction of divalent copper by sugar.*
 - (a) Place 2 ml of tungstic acid 1:10 blood, plasma, or spinal filtrate (par 190) in a special Folin-Wu blood-sugar test tube. (If spinal fluid is analyzed, use only half as much sodium tungstate and sulfuric acid in precipitating the proteins as are used for blood or plasma. This tube has a bulb of slightly less than 4 ml capacity at the bottom, and above the bulb a constricted tube of 8-mm diameter and 40-mm length; above this the tube expands to about 20-mm diameter and is marked to contain 25 ml.
 - (b) In two other Folin-Wu tubes place 2 ml of each of the two working standard solutions.
 - (c) To each of the three tubes add 2 ml of the "colorimetric" alkaline copper tartrate solution. The surface of each solution should now lie within the 8-mm tube above the bulb. The purpose of this constricted tube is to make the surface of contact between the solution and the air small, so that the proportion of reduced Cu^+ that becomes reoxidized by atmospheric air to

Cu^{++} is kept negligibly small. If the size of the bulb is such that the surface of the 4 ml of solution does not lie in the 8-mm tube, discard the tube.

- (d) Immerse the solution-filled parts of the tubes in actively boiling water for exactly 6 minutes. Then transfer the tubes at once to water at room temperature, and let them cool there for about 3 minutes. Do not shake the tubes either while heating or while cooling, or measurable amounts of the reduced copper may be reoxidized by the air.
- (2) *Reduction of molybdate by cuprous oxide.* Add to each tube 2 ml of the phosphomolybdate solution. The cuprous oxide dissolves within 2 or 3 minutes. While waiting for solution of the Cu_2O to become complete, mix in another vessel 1 volume of the phosphomolybdate solution with 4 volumes of water. Add enough of this diluted phosphomolybdate to each Folin-Wu tube to bring the volume to the 25 ml mark. Stopper and mix thoroughly by inverting several times.
- (3) *Colorimetric measurement.* Compare the blood filtrate with whichever of the two standards most nearly matches the filtrate.

Calculation: When the standard with 0.1 mg of glucose per per ml is used the calculation is:

$$\text{Mg sugar per 100 ml blood} = 100 \frac{S}{U}.$$

When the standard with 0.2 mg of glucose per ml is used, calculate:

$$\text{Mg sugar per 100 ml blood} = 200 \frac{S}{U}.$$

S is the reading of the standard, U of the unknown.

d. PROCEDURE FOR PHOTOMETRIC MEASUREMENT.

- (1) *Reduction of cupric copper by sugar.* This step in the procedure is the same as for colorimetric measurement, except that, instead of 2 ml of blood filtrate and 2 ml of "colorimetric" alkaline copper tartrate solution, 1 ml of blood filtrate and 3 ml of "photometric" alkaline tartrate solution are measured into the Folin-Wu tube. The smaller amount of filtrate is taken because otherwise a high blood sugar would give too intense a color for accurate reading in the photometer.
- (2) *Reduction of molybdate by cuprous oxide.* This step is the same as in the colorimetric procedure.

- (3) *Photometric measurement.* The standard is prepared from 1 ml of the working standard with 0.2 mg of sugar per ml. The blank is prepared in the same way as the unknown, but with 1 ml of water in place of the 1 ml of blood filtrate. Light of 520 millimicrons wave length is used.

Calculation:

$$\text{Mg sugar per 100 ml blood} = 200 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown, D_s of the standard. (See par. 157 a (2).)

203. Glucose Tolerance Test

Following the ingestion of a definite amount of glucose, blood sugar is determined at intervals. Urine specimens, taken at the same time as the blood specimens, are tested for glucose and, if positive, the amount of sugar in the urine is determined.

a. **REAGENTS.** Those for blood sugar and urine sugar determination and, in addition, glucose. The dosage used at present is 100 gm regardless of body weight, except in children and in persons differing markedly from normal in stature and general muscular build. It is given in 50 percent solution to the fasting patient. Lemon juice makes the sugar solution more palatable.

b. **PROCEDURE.** Obtain blood and urine specimens on the fasting patient. Give the glucose solution and note the time. One-half hour, 1 hour, 2 hours, and 3 hours after the ingestion of the glucose, take blood and urine specimens. Determine the blood sugar in each specimen and test all urines for glucose. Determine the urinary glucose in any positive specimens.

c. **RESULT.** Record the blood and urine glucose for the fasting $\frac{1}{2}$ -, 1-, 2-, and 3-hour specimens, and also the amount of glucose given.

204. Cholesterol of Plasma or Serum¹³

a. **GENERAL.**

- (1) Cholesterol is an alcohol which exists in plasma partly uncombined ("free cholesterol") and partly esterified with fatty acids ("esterified" or "combined" cholesterol). In the analysis both forms are extracted from plasma by a mixture of acetone and alcohol. The free cholesterol is precipitated from one portion of the extract by means

¹³ Schoenheimer and Sperry. *Journal of Biological Chemistry*, 106, 745 (1939), Modified by Sperry. Unpublished.

of digitonin, which does not precipitate the esterified cholesterol. In another portion of the extract the esterified cholesterol is set free by saponification. Precipitation of this fraction with digitonin then yields "total cholesterol." The cholesterol in each precipitate is measured by the blue-green color formed on treatment with acetic anhydride containing concentrated sulfuric acid (the Lieberman-Burchard reaction). The esterified cholesterol is calculated as the difference between total and free cholesterol.

- (2) Because of the rather weak and transient character of the color, a photometer serves better than a colorimeter for the analysis. The method will first be described for the photometer, then the few modifications will be given which must be made when the final reading is to be made in a Duboscq colorimeter.

b. REAGENTS.

- (1) *Acetone-alcohol mixture.* One volume of redistilled acetone is mixed with 1 volume of *absolute* ethyl alcohol.
- (2) *Ether* (peroxide-free ether).
- (3) *Acetone-ether mixture.* One volume of redistilled acetone is mixed with 2 volumes of peroxide-free ether.
- (4) *Digitonin, 0.4 percent solution.* Dissolve 400 mg of digitonin in 100 ml of distilled water with heating on the steam bath; the solution should be clear. A sediment will develop on standing; remove this by filtration or centrifugation just before use. *A digitonin that gives a markedly opalescent or cloudy solution should not be used in this method. The digitonin must also precipitate cholesterol quantitatively under the conditions described.*
- (5) *Potassium hydroxide solution.* Dissolve 10 gm of pure potassium hydroxide in 20 ml of water. Store the solution in a bottle equipped with a dropping pipette with a rubber bulb attached. If a sediment develops, filter the solution through sintered glass or asbestos before use.
- (6) *Phenolphthalein, 1 percent solution.* Dissolve 1 gm phenolphthalein in 100 ml of ethyl alcohol.
- (7) *Acetic acid, 10 percent solution.* Dilute 10 ml of glacial acetic acid to 100 ml with distilled water.
- (8) *Acetic acid, glacial.* Highest purity reagent grade product is recommended.
- (9) *Acetic anhydride.* (99 to 100 percent, chloride-free.) Highest purity reagent grade. If sediment or color is present, redistill.
- (10) *Sulfuric acid, concentrated.*

- (11) *Cholesterol, stock standard solution.* One hundred mg of cholesterol is dissolved in glacial acetic acid and the volume is brought to 100 ml. The cholesterol used must have been crystallized from an anhydrous solvent, it must be odorless and pure white, and it must melt sharply at not below 147° C. (uncorrected). If these criteria are not met, recrystallize from an anhydrous solvent, such as absolute ethyl or methyl alcohol, or ethylene chloride.
- (12) *Cholesterol, working standard solution, 0.1 mg per ml.* Ten ml of the stock standard solution is diluted to 100 ml with glacial acetic acid.

c. APPARATUS.

- (1) *Filter paper*, Whatman No. 1 or its equivalent, extracted with hot alcohol until completely free of sterols.
- (2) *Stirring rods*, approximately 13 cm long and 3 mm in diameter.
- (3) *Preserving jars*, either pint or quart size, with rubber gaskets.
- (4) *Dropping bottles* for the solvents. The bottles are equipped with ground in pipettes that carry rubber bulbs.
- (5) *Centrifuge tubes*, pyrex, heavy-duty, 12 ml, calibrated roughly at 6 ml, and numbered.
- (6) *Rack* for holding stirring rods. This may be devised from a piece of heavy wire.
- (7) *Dark cabinet*, containing a water bath. A wooden packing box, equipped with a door or curtain, will serve. The cabinet is fitted with a pan or tray, about 10 cm deep, to serve as a water bath. The larger the water bath, the easier it will be to control the temperature. Inlets should be made for a thermometer, and for a funnel for the addition of water, by drilling holes in the top of the box.
- (8) *Metal racks or wire baskets*, divided into compartments by wires, for holding centrifuge and photometer tubes in the water bath.
- (9) *Colorimeter or spectrophotometer.* For the photometer, cuvettes presenting a transmitting length of solution of 15 to 20 mm are desirable, such as the 19-mm test-tube cuvettes of the Coleman Junior spectrophotometer.
- (10) *Stop watch.*

d. PROCEDURE.

- (1) *Extraction.* Place 7 to 10 ml of the acetoneabsolute alcohol solution in a 25 ml volumetric flask and pipette 1 ml of blood serum into the solution, allowing the serum to run down the wall of the flask. Do not agitate during the addition, but swirl the liquid vigorously as soon as

the pipette is withdrawn. A finely divided precipitate should result. Heat the flask over a steam bath, with fairly rapid rotation to prevent bumping, until the solvent boils. Cool the flask to room temperature, add acetone-alcohol to the mark, using a dropping pipette for the final adjustment, mix the contents thoroughly, and filter into a clean flask. The filtrate should be perfectly clear. Pipette samples for the subsequent steps at once to minimize evaporation.

(2) *Precipitation of free cholesterol.*

- (a) (The procedure is described for a single determination. In practice, the average time per determination may be greatly shortened by carrying through a number of analyses together.) Pipette 7 ml of the filtrate into a 15 ml centrifuge tube, record the tube number, add 3.5 ml of the digitonin solution (precise measurement is not necessary) and 1 drop of the 10 percent acetic acid solution, and stir thoroughly with a stirring rod, which is left in the tube; place the tube in a preserving jar, cover it tightly, and leave it overnight at room temperature.
- (b) Transfer the tube to a test-tube rack, and stir the contents to break up clumps of precipitate and to free particles that may adhere to the walls of the tube near the surface of the liquid; remove the rod, and place it on the rack with care so that no precipitate adhering to the rod is lost. If several samples are carried through at the same time, note the position of each rod so it may be returned to the proper tube. Centrifuge the tube until the precipitate is packed tightly enough to permit decantation of the supernatant liquid without loss. A few small particles usually remain suspended and cannot be centrifuged down; their loss does not affect the result. Decant and discard the supernatant solution. After decantation, drain the tube for a few moments and remove the last drop by touching the lip to a clean towel. Replace the stirring rod in the tube, and wash down the wall of the tube and the rod with about 4 ml of the acetone-ether solution, delivered from a dropping pipette; stir the contents thoroughly, return the rod to the rack, centrifuge the tube, and decant the centrifugate as before. Wash the precipitate twice more in the same manner, but with ether instead of acetone-ether. Replace the rod and set the tube aside until ready for color development. The

sample may be stored for several days at this stage. If color development is to follow at once, be sure that the ether has evaporated.

(3) *Precipitation of total cholesterol.*

- (a) *Saponification.* Add 3 drops of the potassium hydroxide solution to a 15 ml centrifuge tube, that is marked to contain 6 ml (approximately). Pipette 3 ml of the blood filtrate into the tube, record the tube number, and stir at intervals with a vigorous up and down motion of the stirring rod until no droplets of the alkali can be seen at the tip of the tube. Leave the rod in the tube. Place a layer of sand about 5 cm deep in a preserving jar, and heat it in a water bath until the temperature of the sand is about 45° C. Place the tube in the sand, cover the jar tightly, and put it in an incubator at 37 to 40° C. for 30 minutes.
 - (b) *Neutralization.* Remove the tube to a rack, allow the tube to cool, raise the stirring rod and add alcohol-acetone to the 6 ml mark; add 1 drop of phenolphthalein solution, and titrate with the 10 percent acetic acid solution. About 0.4 ml should be required. Add 1 drop more than enough to decolorize the phenolphthalein.
 - (c) *Precipitation.* Add 3 ml of the digitonin solution, stir thoroughly, place the tube in a preserving jar, cover it tightly, and leave it at room temperature for at least 3 hours, preferably overnight. Carry out the centrifuging and washing as described for free cholesterol, except that only one ether washing is necessary.
- (4) *Drying of cholesterol digitonide.* Heat a shallow pan containing a layer of sand about 3 cm deep to 110 to 115° C. in an oven. Place the tubes containing the precipitates, of either free or total cholesterol, in the sand in the order in which readings are to be taken, and return the pan to the oven for 30 minutes.
- (5) *Resolution of cholesterol digitonide in glacial acetic acid.*
- (a) While the precipitates are drying in the oven adjust the darkened water bath to a temperature at which it can be maintained during the rest of the procedure by adding hot or cold water as needed. The most satisfactory bath temperature is 25°, but if the laboratory temperature is higher, and cold water not available, the bath may be set at 30°, 35°, or 40°.
 - (b) After the 30-minute drying, remove the sand bath containing the tubes from the oven and pipette 2 ml of

glacial acetic acid into the first tube while it is still in the hot sand. Allow the acid to wash down the rod and the wall of the tube. Stir the contents vigorously and leave the tube, containing the rod, in the sand while acid is being added to the next two or three tubes, a total of 2 or 3 minutes. Stir again, remove the tube from the sand, allow it to cool, and place it in a rack in the water bath.

- (c) In the same manner add acetic acid to all of the tubes in the series, and place them in the water bath.
- (6) *Preparation of standards.* Include with each series of determinations at least two standards prepared as follows: Pipette 2 ml portions of the standard solution of cholesterol, containing 0.1 mg per ml, into centrifuge tubes, insert stirring rods, and place the tubes in the water bath along with the unknown samples.
- (7) *Preparation of acetic anhydride-sulfuric acid reagent.*
 - (a) This reagent decomposes on standing, and must be made up fresh for each series of determinations. If the reagent is kept in an ice-bath it can be used for 1 hour, but *if it is prepared and kept at room temperature it must all be used within at most 30 minutes.* Otherwise the reagent weakens, so that development of color with cholesterol is slower than indicated in the directions below, and the final color may be weaker.
 - (b) Measure from a burette enough acetic anhydride for the series of analyses (4 ml for each analysis) into a glass-stoppered flask large enough to hold at least twice the volume of fluid added.
 - (c) If ice is available, chill the flask in ice water. To the chilled anhydride add concentrated sulfuric acid slowly, 1 ml for each 20 ml of the anhydride. Take 2 minutes for the addition and during it rotate the flask in the bath. The temperature rises several degrees despite the bath. After the addition, remove the flask from the ice bath, stopper it, and shake vigorously for a few seconds, then return it to the ice bath and let it stand there 10 minutes to cool before beginning to use it. Keep the flask in the ice bath until finished with its use. Except during withdrawal of portions of the reagent, keep the flask closed in order to prevent absorption of atmospheric moisture by the reagent. If ice is not available a bath at room temperature may be used. The sulfuric acid is added in the manner described above. A thermometer is kept in the mixture,

and after the addition of the sulfuric acid is completed, rotation in the bath is continued until the reagent temperature has fallen to within 1° C. of bath temperature. The flask is then kept stoppered, except when reagent is withdrawn.

- (8) *Preparation of blank.* In a photometer cuvette mix 2 ml of glacial acetic acid and 4 ml of the anhydride-sulfuric acid reagent, and place the cuvette in the water bath with the tubes of redissolved digitonide.
- (9) *Development of color in standards and unknowns.*
 - (a) *Conditions for color development.* Four ml of the anhydride-sulfuric acid reagent is added to each tube of redissolved cholesterol digitonide, and the tube is kept in the darkened water bath at 25° C. (or at higher temperature if the bath can not be kept at 25°) until time for the photometric reading. The color develops gradually, reaches a maximum, and soon begins to fade. The time, from addition of the anhydride-sulfuric acid reagent until fading begins, depends on the temperature of the bath in which the tubes of reacting solution stand. At 25° the time is 40 minutes; at 30° , 23 minutes; at 35° , 15 minutes; at 40° , 11 minutes. Of each period, about four-fifths of the time is taken to develop the maximum color, which endures for the last fifth. The moment at the end of the interval of maximal color is chosen for the reading, because the result at that moment is less likely to be influenced by variations in the reagent than at the beginning of the interval of maximal color.
 - (b) *Development of color in a series of determinations.* When a series of analyses are made together, arrange the centrifuge tubes in the darkened water bath in the order in which they are to be read, with one standard at the beginning of the series and one at the end. Into tube No. 1 of the series (one of the standards) pipette 4 ml of the acetic anhydride-sulfuric acid reagent, and note the exact time, or, preferably start a stop watch. Stir vigorously with the rod to insure immediate and complete mixing. Then pour the entire solution into a clean, dry photometer cuvette, marked No. 1, and place the cuvette back in the bath in the place from which the centrifuge tube was taken.
 - (c) At definite, uniform time intervals, such as 2 minutes, tubes No. 2, No. 3, etc. of the series are successively treated in the same manner, and their contents trans-

ferred to similarly numbered cuvettes, which are placed in the bath. The time interval used between additions of the anhydride-sulfuric acid reagent to the successive tubes must be long enough to permit one to complete easily the addition of reagent, mixing, and transfer to a cuvette. The interval must also permit one, after color is developed, to read the optical density of each cholesterol solution and then recheck the zero point with the blank before reading the next solution. Intervals of 2 minutes usually suffice, and with practice the interval may be shortened to 1 minute.

e. PHOTOMETRIC MEASUREMENT.

- (1) While the color is developing in the cholesterol-containing cuvettes, remove the cuvette with the blank solution from the water bath, quickly wipe off the water with a clean, dry towel, then dip the tube into alcohol and wipe again. Place the cuvette in the photometer and adjust the galvanometer to read zero optical density with the wave length set at 625 millimicrons. Then return the blank cuvette to the bath.
- (2) One minute before the end of the reaction period of cholesterol solution No. 1 (39 minutes after adding the anhydride-sulfuric acid reagent if the bath is at 25°) remove cuvette No. 1 (one of the standard solutions), wipe and dry it as described for the blank, and read the optical density. Be careful not to stir up the small quantity of sediment that may be left in the bottom of the cuvette.
- (3) After reading No. 1, recheck the zero with the blank and adjust the galvanometer if necessary. Then read No. 2 in the same manner. If the time intervals at which the anhydride-sulfuric acid was added were 2 minutes, the successive cholesterol-containing cuvettes are taken from the 25° bath when the stop watch registers 39, 41, 43, etc., minutes. If the bath temperature is 30°, the cuvettes are taken when the stop watch registers, 22, 24, 26, etc., minutes. The reading of each cuvette is taken 1 minute after withdrawal from the bath. *Exact timing according to temperature is necessary for exact results.*
- (4) Continue in this way until all the tubes of the series have been read, the zero point being checked with the blank before each reading.
- (5) The intervals between readings must be the same as between additions of the anhydride-sulfuric acid reagent,

so that the reaction period between the addition and the reading will be the same for each cuvette.

- (6) *Photometric calculation.* When a sample of the size prescribed above is used the calculations are made by the following equations:

$$\text{Mg total cholesterol per 100 ml serum } (C_t) = \frac{166.7 D_u}{D_s}.$$

$$\text{Mg free cholesterol per 100 ml serum } (C_f) = \frac{71.4 D_u}{D_s}.$$

$$\text{Mg combined cholesterol per 100 ml serum } (C_c) = C_t - C_f.$$

$$\text{Percentage of cholesterol combined} = \frac{100 C_c}{C_t}.$$

D_s is the optical density of the standard, D_u that of the unknown.

- (7) In cases in which extremely high cholesterol contents make it necessary to take smaller samples than those prescribed for the usual procedure, the values calculated by the above equations are multiplied by 1.5, 2, or 3, as directed in *g* below.

f. COLORIMETRIC MEASUREMENT. The analysis is the same as with the photometer, except for the following changes:

- (1) *Working standards.* Standards are used containing 0.07, 0.12, and 0.30 mg of cholesterol per ml. These are made by measuring 7, 12, and 30 ml, respectively, of the stock standard (1 mg per ml) into 100 ml flasks, and diluting each to the mark with glacial acetic acid.
- (2) *Development of color.*
 - (a) Because of the transient nature of the color, and the fact that standard and unknown must be observed at the same time, a standard is prepared simultaneously with each unknown.
 - (b) For free cholesterol 2 ml of the standard containing 0.07 mg per ml is ordinarily used.
 - (c) For total cholesterol 2 ml of the standard containing 0.12 mg per ml is ordinarily used.
 - (d) For total cholesterol exceeding 300 mg per 100 ml of plasma, 2 ml of the standard containing 0.3 mg per ml is used. With practice one can judge from the bulk of the digitonide precipitate when so much cholesterol is present that the standard containing 0.3 mg per ml is needed.

- (e) The acetic anhydride-sulfuric acid reagent is added to each unknown and its accompanying standard at as nearly as possible the same time. The color is developed in the centrifuge tubes, and the solutions are transferred to colorimeter cups just before readings are to be made.
- (f) When analyses are done in series, 10 minutes are let pass, after adding the acetic anhydride-sulfuric acid reagent to each unknown standard pair, before the reagent is added to the next pair in the series. The 10-minute intervals between readings in a series permit one to wash and dry the colorimeter cups after each reading.
- (g) After each reading the cups are washed with alcohol and ether and dried; there is not enough cholesterol solution to permit using part of it to wash the cups.
- (h) The duration of the reaction with the acetic anhydride-sulfuric acid reagent is the same as for the photometric measurement.
- (i) The cups and plungers must be scrupulously dried before the cholesterol solutions are added, because the color is affected by small quantities of water.
- (j) *Calculations from colorimeter readings:*

$$\text{Mg total cholesterol per 100 ml serum } (C_t) = 1,667 C_s \times \frac{S}{U}.$$

$$\text{Mg free cholesterol per 100 ml serum } (C_f) = 714 C_s \times \frac{S}{U}.$$

$$\text{Mg combined cholesterol per 100 ml serum } (C_c) = C_t - C_f.$$

$$\text{Percentage of cholesterol combined} = \frac{100 C_c}{C_t}.$$

C_s is the mg of cholesterol per ml in the standard solution used; S is the reading of the standard, U that of the unknown.

- (k) The above calculations hold when samples of the size prescribed above for usual use are used. When high cholesterol contents necessitate smaller samples, the values calculated by the above equations are multiplied by 1.5, 2, or 3, as directed below under *g*.

g. NOTES AND PRECAUTIONS.

- (1) *Total cholesterol.* The concentration of total cholesterol in the serum of healthy persons varies from a little over

100 to nearly 400 mg per 100 ml; in disease it may reach 1,000 or more. The method as given covers concentration ranges from about 110 to 700 mg per 100 ml, which are within the desirable range of the photometer (10 to 70 percent transmittance). If concentrations above 700 mg per 100 ml are encountered, they may be handled in either of two ways, (a) or (b):

- (a) For "extraction" take only 0.5 ml of serum in 25 ml, but otherwise follow the procedure exactly as given. In calculating multiply by 2 the values and free cholesterol given by the equations.
- (b) For "precipitation of total cholesterol" take, instead of 3 ml of filtrate, 2 ml, or in extreme hypercholesteremia only 1 ml and decrease the volume of KOH solution used for saponification from 3 drops to 2 or 1 drop, respectively. In calculating, multiply the value for total cholesterol given by the equation by 1.5 for 2 ml of filtrate, or by 3 for 1 ml of filtrate.

(2) *Free cholesterol.*

- (a) The method as given covers concentrations of free cholesterol from about 45 to 300 mg per 100 ml of serum, with readings which are within the desirable range of the photometer. Values above 300 mg are rare; they may be handled by taking for "extraction" 0.5 ml, instead of 1 ml, of serum, and multiplying by 2 the value for free cholesterol given by the equation.
 - (b) For free cholesterol values below 45 mg per 100 ml of serum there is no way of obtaining color densities great enough to be within the desirable range, without drastic revision of the method. Do not increase the proportion of serum extracted above 1 ml, as the extraction may then be incomplete. One must accept the necessity of measuring an undesirably low optical density, and make the measurement with as much care as possible.
- (3) *Whole blood.* The method may be applied to the determination of cholesterol in whole blood, but this is inadvisable. The cholesterol of red cells is entirely in the free form, and the concentration is maintained within a narrow range in all conditions of health and disease that have been investigated. There is no evidence of any direct relation between the cholesterol of the serum and that of the red cells, and the determination of both together in whole blood is not an accurate measure of the amount in either of them.

(4) *Centrifuging.*

- (a) Considerable time may be saved in centrifuging by selecting centrifuge tubes of the same weight within the tolerance allowed in balancing the centrifuge. These tubes should be calibrated and numbered, and kept for exclusive use in cholesterol determinations. With such balanced tubes it is not necessary to balance tubes and carriers before each centrifuging if the carriers are balanced at the start; but be careful not to centrifuge "free" opposite "total" samples, since the volumes are different.
- (b) The time necessary for centrifuging depends on the characteristics of the centrifuge used and must be determined by the operator. In decanting, pour slowly and be sure that no precipitate is loosened and carried out as the solution draws away from the packed precipitate. With a radius of 13.5 cm and a speed of 2,800 rpm, 15 minutes centrifuging is adequate for "free" samples at the first stage; 5 to 7 minutes suffice for "total" samples and for the acetone-ether and ether washings.
- (5) *Bookkeeping.* When a large number of samples are carried through the procedure together, careful bookkeeping is essential to avoid intermixing of tubes and placing stirring rods in the wrong tubes. At each stage, place the tubes in racks in the same order in which they are entered in the record and read them in that order.
- (6) *Exclusion of water.* The reaction which produces the color is grossly affected by small quantities of water. Be careful that water does not drip into any tube while tubes are being removed from the bath and avoid splashing, if the bath is transported from one place to another.

205. Bilirubin of Serum (van den Bergh diazo reaction)

a. GENERAL. Bilirubin, like aromatic amines, combines with diazonium salts to form azo dyes. Van den Bergh applied the reaction to serum, forming "azorubin" by coupling diazotized sulfanilic acid with the bilirubin, and measuring the azorubin colorimetrically. The color is less interfered with by other serum pigments than is the color of the "icterus index" measurement, and provides a more accurate measurement of the serum bilirubin. In normal serum and in sera from many cases of jaundice, particularly hemolytic jaundice, the bilirubin appears to be attached to the serum proteins, and does not begin to show the azorubin color in water solution until several minutes after the diazo reagent has

been added; this delayed reaction is called the "indirect van den Bergh." Particularly in cases of obstructive jaundice, however, a considerable part of the serum bilirubin is free to react at once with the diazo reagent; this quick reaction is called the "direct van den Bergh." Addition of alcohol sets all the bilirubin free to react with the diazo reagent, and is used to make the reaction quantitative for total bilirubin content of the serum.

b. REAGENTS.

- (1) *Methyl alcohol, absolute.*
- (2) *Sulfanilic acid, 0.1 percent solution.* Dissolve 1 gm of sulfanilic acid in 15 ml of concentrated HCl and dilute to 1 liter with distilled water.
- (3) *Sodium nitrite, 0.5 percent solution.* Dissolve 0.5 gm of NaNO_2 in a little water and dilute to 100 ml. The solution should be prepared on the day it is used.
- (4) *Diazo reagent.* Add 0.3 ml of the nitrite solution to 10 ml of the sulfanilic acid solution. The diazo reagent is prepared within a few minutes of the time when it is to be used.
- (5) *Hydrochloric acid, dilute.* Dilute 15 ml of concentrated HCl to 1 liter with distilled water.
- (6) *Bilirubin, stock standard.* Dissolve 40 mg of bilirubin in pure chloroform in a 100 ml flask, and dilute to the mark with chloroform. Store in a brown glass bottle closed with a well-ground stopper to prevent evaporation. The solution is stable.
- (7) *Bilirubin, dilute working standard, 0.4 mg per 100 ml.* Dilute 1 ml of the stock standard to 100 ml with absolute methyl alcohol.

c. HANDLING OF BLOOD. To avoid turbidity of serum caused by alimentary lipemia, the blood is taken when the patient is in the post-absorptive state. The blood is allowed to clot, and is centrifuged, after the serum has separated, with every precaution to avoid hemolysis. (See par. 188.) The diazo reaction must be applied within 2 hours after the blood is collected. If even the separated serum is allowed to stand, the reaction becomes altered. Sera which, when fresh, give direct reactions may, after standing, give only indirect reactions.

d. QUALITATIVE REACTION.¹⁴

- (1) *Dilution of serum.* Jaundiced sera must be diluted with water before adding the diazo reagent. The proper dilution can be judged from the intensity of the yellow color of the serum. As a rule a fivefold or tenfold dilution is satisfactory.

¹⁴ Sepulveda and Osterberg, *Journal of Laboratory and Clinical Medicine*, 28, 1359 (1943).

- (2) *Procedure.* To 1 ml of the diluted serum, add 2 ml of fresh diazo reagent and mix. Three results may be given:
- (a) *Direct reaction.* The color change is detected as soon as serum and diazo reagent are mixed; it reaches maximum intensity in from 1 to 2 minutes. The color is reddish-pink in undiluted serum, and purplish or reddish-violet in diluted serum.
 - (b) *Indirect reaction.* No change in color is noted during the first 2 minutes. After 4 to 10 minutes a golden color appears; the color then slowly changes to a brownish-red, and generally at the end of 1 or 2 hours the pink color of azorubin has developed.
 - (c) *Biphasic reaction.* This is rare. The color appears during the first 2 minutes; it is not the typical pink, but a brownish-red, which reaches its maximum intensity in about 5 minutes.
- e. QUANTITATIVE SERUM BILIRUBIN.¹⁵
- (1) *Dilution of serum.*
 - (a) *Serum with less than 10 mg of bilirubin per 100 ml.* Dilute 1 ml of serum to 10 ml with distilled water and mix. This serves for all except extremely jaundiced sera.
 - (b) *Serum with more than 10 mg of bilirubin per 100 ml.* Dilute 1 ml of serum to 25 ml with distilled water and mix.
 - (2) *Preparation of receivers for standard, blank, and serum.*
 - (a) *Standard.* Pipette 5 ml of the working standard solution and 1 ml of freshly prepared diazo reagent into a test-tube or flask of about 25 ml capacity (a colorimeter cup or photometer cuvette may be used).
 - (b) *Blank* (required for photometric but not for colorimetric measurement). Pipette 5 ml of absolute methyl alcohol and 1 ml of the dilute hydrochloric acid into a similar receiver (diazo reagent is omitted).
 - (c) *Serum.* Pipette 5 ml of absolute methyl alcohol and 1 ml of fresh diazo reagent into a similar receiver.
 - (3) *Color development.* To the receiver for the *standard* add 4 ml of distilled water. To the receiver for the *blank* and that for the *serum* add 4 ml of the diluted serum. (Addition of serum to the photometric blank makes the blank embody a correction for light absorption by colored serum constituents other than bilirubin, and by any turbidity that may develop when the diluted serum is mixed with methyl alcohol and hydrochloric acid.) Mix the

¹⁵ Evelyn and Malloy, *Journal of Biological Chemistry*, 119, 491 (1937).

contents of each receiver, and let stand 30 minutes at room temperature for the azorubin color to develop.

- (4) *Colorimetric measurement.* The serum is compared with the standard. Before placing the colorimeter cups in position detach any bubbles that may be present by tapping the cups against a wooden block. (Bubbles of nitric oxide gas are likely to form from decomposition of the nitrous acid in the diazo reagent.)

Calculation: When the serum is diluted tenfold the calculation is:

$$\text{Mg bilirubin per 100 ml serum} = 5 \times \frac{S}{U}.$$

When the serum is diluted twenty-five fold the calculation is:

$$\text{Mg bilirubin per 100 ml serum} = 12.5 \times \frac{S}{U}.$$

S is the reading of the standard, U that of the unknown (serum).

- (5) *Photometric measurement.*

- (a) Light of wave length 530 to 540 millimicrons is used. The blank is likely to vary appreciably from serum to serum, because it compensates for light absorption by turbidity and serum pigments. Because of this variability it is convenient, when running a series of analyses, to set the scale for zero optical density with a water blank, and to measure the density of each serum blank compared with water as zero, rather than to follow the usually preferable procedure of setting the zero point with the reagent blank. Hence, *with the zero set with a water blank*, the readings of the different serum blanks of the respective diazotized serums, and of the standard are taken.

Calculation:

$$\text{Mg bilirubin per 100 ml serum} = 5 \times \frac{D_u - D_b}{D_s}$$

when the serum is diluted tenfold.

When the serum is diluted twenty-five-fold the calculation is:

$$\text{Mg bilirubin per 100 ml serum} = 12.5 \times \frac{D_u - D_b}{D_s}.$$

D_u is the optical density of the unknown (serum), D_b the density of the blank, and D_s the density of the standard.

- (b) The reading of D_s has been found by Evelyn and Malloy to be sufficiently constant to permit using a constant value for it, without redetermining it in each analysis. If a constant value of D_s is used, and k is taken as $5/D_s$ for tenfold diluted serum, $12.5/D_s$ for twenty-five-fold diluted serum, the calculation becomes:

$$\text{Mg bilirubin per 100 ml serum} = k (D_s - D_s)$$
- (6) *Use of cobaltous sulfate standard solution* (MacLay and Osterberg, Staff Meetings of the Mayo Clinic (1944)).
- (a) When bilirubin is not available for standards, cobaltous sulfate may be substituted. A water solution of 600 mg of cobaltous sulfate ($\text{CoSO}_4 \cdot 6\text{H}_2\text{O}$) per 100 ml has the same optical density as a diazotized bilirubin solution containing 0.1 mg of bilirubin per 100 ml when light filtered through a No. 2 Cenco filter (transmission range 550 to 700 millimicrons) is used in a photometer. In a Duboscq colorimeter, a green glass filter (Wratten No. 74) over the eyepiece overcomes the slight disparity in color between the cobaltous sulfate and the diazotized bilirubin.
- (b) When the serum is diluted tenfold the factor 2.5 replaces the factor 5 in the equations given for both colorimetric and photometric calculations above. If the dilution is twenty-five-fold, the factor 6.25 replaces 12.5 in the equation for that dilution. In case a fifty-fold dilution is used, the factor is 12.5 for use with the cobaltous sulfate standard.
- (c) If the reading with the tenfold diluted serum indicates more than 5 mg of bilirubin per 100 ml, repeat the determination, diluting the serum to twenty-five-fold or fiftyfold. Comparison with the cobaltous sulfate standard is not accurate if the bilirubin concentration in the *diluted* serum exceeds 0.5 mg per 100 ml.

206. Icterus Index of Serum¹⁶

a. GENERAL. The icterus index is a rough measure of the bile pigments in the serum. It is carried out by comparing the color of the serum with that of a standard solution of potassium dichromate. It is simpler, but less accurate, than the van den Bergh diazo reaction (par. 205) for measurement of the bilirubin content of serum. If the icterus index is within normal limits, the result may be accepted. If the index is high, however, there is a possibility that the high value may be due to lipochrome pigments

¹⁶ Meulengracht, *Deutsche Archives f. klinische Medecin*, 132, 285 (1920).

or to carotinemia; in the latter condition the index may be as high as in severe jaundice. It is convenient practice to run routine icterus indices and confirm high values by checking them with the van den Bergh determination.

b. PREPARATION OF SUBJECT AND SERUM.

- (1) To avoid carotinemia the subject should not eat carrots for 24 hours before the test.
- (2) If serum is lipemic the turbidity makes the readings too high, especially if they are made with a photometer. To avoid lipemia the blood is drawn 4 or more hours after ingestion of food. If this precaution does not suffice to yield a serum that is clear and remains clear when diluted, the serum can not be used for a photometric determination of the index. (In some diseases, such as nephrosis and severe diabetes, there may be chronic lipemia.)
- (3) The slightest trace of hemolysis vitiates the results and must be avoided. The needle and syringe used to draw the blood must be dry. The blood is allowed to clot in a dry centrifuge tube protected from light, and is then centrifuged.

c. REAGENTS.

- (1) *Potassium dichromate, 0.01 percent standard solution.* Dissolve 100 mg of $K_2Cr_2O_7$ in about 500 ml of water in a 1-liter flask. Add 4 drops of concentrated sulfuric acid, and dilute to the mark with water. Keep in a dark glass bottle.
- (2) *Sodium chloride, 0.9 percent solution.*
- (3) *Sodium citrate, 5 percent solution.*

d. PROCEDURE.

- (1) *Colorimetric measurement.* If the serum is more deeply dyed than the standard dichromate solution, dilute the serum with accurately measured volumes of the 0.9 percent NaCl solution until the diluted serum has about the same color as the standard. The necessary dilution may be to 2, 5, 10, or even more times the original volume of the serum. Place the diluted serum in one cup and the dichromate solution in the other cup, of the colorimeter.

Calculation: The icterus index is defined as unity when the color of the undiluted serum matches that of the 0.01 percent dichromate solution. Hence the calculation formula is:

$$\text{Icterus index} = \frac{S \times V}{U}.$$

S is the reading of the standard, U is the reading of the unknown, V the number of volumes to which 1 volume of serum is diluted before comparison with the dichromate standard is made. V is 1 when no dilution is made.

- (2) *Photometric measurement.* Dilute 1 ml of clear serum to 10 ml with 5 percent citrate solution and mix. Read in the photometer with light of 420 millimicrons wave length. Use a 5 percent sodium citrate solution as the blank. The optical density of the 0.01 percent $K_2Cr_2O_7$ solution is read with the same wave length, with the zero set with a water blank.

Calculation:

$$Icterus\ index = 10 \times \frac{D\ of\ serum}{D\ of\ dichromate}.$$

D of serum is the optical density of the tenfold diluted serum. D of dichromate the density of the 0.01 percent $K_2Cr_2O_7$ solution.

If the icteric index is found to be over 50, repeat the analysis, but with a greater than 1:10 dilution of the serum. If V represents the number of volumes to which 1 volume of serum is diluted, the calculation is:

$$Icterus\ index = V \times \frac{D\ of\ diluted\ serum}{D\ of\ dichromate}.$$

Once the value of D of dichromate has been established for a particular photometer, cuvette, and wave length, it is valid indefinitely, and need not be checked with each analysis.

207. Thymol Turbidity Test

a. GENERAL. Serum from patients with liver disease causes turbidity in a buffered thymol solution. This reaction is thought to be due to an abnormal globulin which forms a globulin-thymol-lipoid complex. The test is performed with serum which is not inactivated and results are reported in arbitrary units. The normal range is 0 to 4 units.

b. REAGENTS.

- (1) *Buffered thymol solution* — pH 7.8. Place 1.03 gm of sodium barbital in a liter Florence flask and add 1.38 gm of barbital and 3 gm of pure powdered thymol crystals. Add 500 ml of distilled water and heat just to the boiling point; shake well and cool to room temperature. The solution should be turbid. Add a small amount of powdered thymol crystals and allow to stand overnight at

room temperature. Shake well and then filter the clear solution from the crystalline deposit. Keep in a glass-stoppered bottle at room temperature. If the solution becomes turbid on standing, a fresh solution should be prepared.

- (2) *Stock standard barium sulfate solution.* To prepare barium sulfate standards, dissolve 1 gm of anhydrous barium chloride (or 1.173 gms of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) in 100 ml of water. Bring this solution and a 0.2 normal sulfuric acid solution to a temperature of 10°C . Transfer 3 ml of the barium chloride solution to a 100 ml volumetric flask and make up to volume with the 0.2 normal sulfuric acid; mix well. This stock suspension settles on standing, but may be resuspended when needed. If 1 volume of the stock suspension is mixed with 21.22 volumes of 0.2 normal sulfuric acid, the turbidity is equivalent to 1 unit in MacLagan's standards. Similarly, when 1 volume of stock suspension is mixed with 10.11, 6.41, 4.55, 3.44, 2.70, 2.17, 1.78, 1.47, 1.22, 0.48, 0.11 volumes of 0.2 normal sulfuric acid the resulting mixtures represent 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, and 20 units respectively.

c. *PROCEDURE.* Place 0.1 ml of serum in a cuvette and add 6.0 ml of the buffered thymol solution. Mix well by rotation, allow to stand for 30 minutes at room temperature and compare the turbidity with that of the standard barium sulfate dilutions. The turbidity may be measured in a spectrophotometer using a wave length of 650 millimicrons and using the buffered thymol solution as a blank. The turbidity may be expressed in units derived from a curve or table of values prepared by use of the barium sulfate suspensions. Use distilled water as the blank and a wave length of 650 millimicrons. Read each tube immediately after thorough shaking and plot on semilogarithmic paper. The results should be a straight line. Recalibration will be necessary from time to time.

d. *DISCUSSION.* Normal sera in the presence of this reagent have turbidity ratings of 0 to 4 units. Sera from patients with severe liver dysfunction may have ratings as high as 30 to 35 units but rarely above 20. When the test is positive, flocculation will often occur on standing longer than 30 minutes; hence the turbidity should be measured after an interval of 30 minutes and not at some later time.

The pH of the buffered thymol solution should be checked with a pH meter since it will vary with different lots of sodium barbital. If the pH is too alkaline, make a second solution omitting the sodium barbital. This solution will be acid. Add 0.1 ml portions of this second solution to 10.0 ml of the first solution until the

correct pH is obtained, checking the pH between each addition with nitrazine paper and making a final check with the pH meter. Calculate the amount of the second solution needed to adjust the pH of the first solution. Add this amount to the first solution and check the pH again with a pH meter.

If the original solution is too acid, make a fresh solution using a different lot of sodium barbital.

A pH of 7.8 was used in the original description by MacLagan but some authors state that a pH of 7.55 gives greater sensitivity without giving false positives.

208. Cephalin-Cholesterol Flocculation Test

a. Serum from patients with liver disease will often cause flocculation of a colloidal suspension of cephalin-cholesterol. This flocculation is due to increase of gamma globulin, decrease of albumin or decrease of the flocculation inhibiting powers of the albumin fraction of the serum. Such changes in serum are usually associated with disease of the liver parenchyma.

b. The specimen should be tested within a few hours after collection. Bacterial contamination may give a false flocculation. Glassware used in the test must be scrupulously clean and free of acid or heavy metals.

c. To prepare cephalin-cholesterol emulsion, pipette 35 ml of distilled water into a graduated centrifuge tube and heat on a water bath to 60 to 75° C. Add, with stirring, 1 ml of stock cephalin-cholesterol solution and allow to simmer on boiling water bath until volume is reduced to 30 ml. Cool before using. This emulsion should be prepared fresh each day.

d. Place 4 ml of 0.85 percent NaCl solution in each of two conical centrifuge tubes and add to one of the tubes 0.2 ml of the patient's serum. The other tube serves as a control. Add 1.0 ml of the cephalin-cholesterol emulsion to each tube and rotate the tubes between the hands to mix. Stopper and allow the tubes to stand at room temperature in the dark. Read at 24 and 48 hours.

e. The results are reported as follows:

- (1) *Negative*. A milky solution with no flocculation.
- (2) *One plus*. Slight flocculation.
- (3) *Two plus*. Moderate flocculation.
- (4) *Three plus*. Marked flocculation.
- (5) *Four plus*. Complete flocculation with clear supernatant fluid.

209. Chloride of Plasma, Serum, or Spinal Fluid¹⁷

a. GENERAL. Chloride solution (plasma or serum) is shaken for 30 or 40 seconds with pulverized silver iodate in dilute phosphoric acid, and iodate dissolved by the reaction, $\text{AgIO}_3 + \text{Cl}^- \rightarrow \text{AgCl} + \text{IO}_3^-$, is titrated with thiosulfate. (See sec. VI, ch. 3.) The reaction is 99.7 percent complete under the conditions fixed by Sendroy. Simultaneously with this reaction the plasma proteins are precipitated by tungstate which is added with the phosphoric acid, and a water-clear, protein-free filtrate is obtained for the titration. No correction for the solubility of the silver iodate is required, because the IO_3^- in solution depresses the solubility of the AgIO_3 to a negligible value at the end of the reaction.

b. APPARATUS. A 25-ml burette, accurate transfer pipettes of 1, 10, and 25-ml capacity, and 50-ml Erlenmeyer flasks are required. In addition either centrifuge tubes of 15- or 50-ml capacity, or else funnels of 5- or 6-cm diameter.

c. REAGENTS.

- (1) *Phosphoric-tungstic acid solution.* Dissolve 6 gm of chloride-free sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) (A.C.S. grade containing less than 0.01 percent of chloride) in 1 liter of water and add 10 ml of concentrated phosphoric acid (sp. gr. 1.72). (If chloride-free sodium tungstate can not be obtained, 2 gm of picric acid may be substituted for the 6 gm of tungstate.)
- (2) *Sodium iodide solution.* Fifty gm of sodium iodide are dissolved in 50 ml of water. The solution is kept in a brown glass bottle with a stopper that is provided with a dropping pipette. The solution is tested for the absence of free iodine by adding 1 ml of the iodide solution to 10 ml of the phosphoric-tungstic acid solution and adding a drop of starch indicator solution. The mixture should remain colorless. If a blue color appears, discard the sodium iodide solution and prepare a fresh one. The solution will keep for several weeks without forming free iodine if kept in a bottle of brown glass which is not exposed to sunlight. In place of the iodide solution, one may use sodium iodide in solid form. The solution is more convenient if many analyses are to be done. Even the solid must be kept in brown glass to avoid oxidation of the surface with formation of free iodine.
- (3) *Starch solution, 1 percent.* (See par. 143.)

¹⁷ Sendroy, *Journal of Biological Chemistry*, 120, 405 (1937), modified by Van Slyke and Hiller, Peters and Van Slyke's methods 1943 edition, page 839.

- (4) *Potassium iodate, 0.1 N solution.* Dissolve 3.567 gm of reagent grade potassium iodate (KIO_3) in water in a 1-liter volumetric flask, dilute to the mark and mix.
- (5) *Sodium thiosulfate, stock 0.9232 N solution.* Dissolve 57.30 gm of crystalline sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) in water and dilute to 250 ml in a volumetric flask. The solution keeps indefinitely. Its concentration is tested and standardized as described in the next paragraph.
- (6) *Sodium thiosulfate, 0.02308 N solution.*
- (a) Transfer with an accurate pipette 25 ml of the 0.9232 N thiosulfate solution to a 1-liter volumetric flask, add 1 gm of borax as a preservative, and dilute to 1 liter.
 - (b) The accuracy of the solution is checked by titrating against the 0.1 N KIO_3 as follows: 5 ml of the 0.1 N KIO_3 , measured from a calibrated transfer pipette, is mixed with 20 ml of water and 5 ml of 1 N H_2SO_4 in a 100 ml Erlenmeyer flask. Of the sodium iodide solution 1.5 ml is added, and the 0.02308 N thiosulfate is run in from a 25-ml burette until the iodine color nearly disappears. Then 3 drops of starch solution are added and the titration is continued until the solution becomes colorless. The volume of thiosulfate required should be 21.67 ml. If it differs by more than 0.1 ml from this, add either water or sodium thiosulfate in calculated amount to the stock 0.9232 N solution.
 - (c) The 0.02308 N solution may change under laboratory conditions at the rate of 1 or 2 parts per thousand per month. Hence it is desirable to restandardize the solution once a month against the 0.1 N KIO_3 . When the volume of KIO_3 solution required in the titration changes by more than 0.1 ml, the 0.02308 N thiosulfate solution is discarded and a fresh solution prepared from the stock solution. The normality, 0.02308, is used because it simplifies calculation, since each 0.1 ml of this thiosulfate used in the titration of plasma chloride indicates 1 millimole of chloride per liter of plasma.
- (7) *Precipitated silver iodate, free from both other iodates and other silver salts.* The AgIO_3 may be tested by shaking 0.5 gm in 25 ml of the phosphoric-tungstic acid solution for a minute, filtering, and titrating the dissolved iodate in 10 ml of filtrate with the 0.02308 N thiosulfate, as described below for titration of plasma filtrates. The volume of thiosulfate used should be 0.50 ml if the tem-

perature is 20°, 1.00 ml if the temperature is 40°, the intermediate variation being approximately linear. If more thiosulfate is required than corresponds to the temperature the AgIO_3 contains other iodates in significant amount. It can be purified by stirring it up in 20 times its weight of water and washing it on a Buchner funnel with water.¹⁸

d. PROCEDURE.

- (1) *Reaction with silver iodate.* One ml of plasma, serum, or spinal fluid is measured from an accurate pipette into a 50 ml centrifuge tube, test tube, or Erlenmeyer flask, and 25 ml of the phosphoric-tungstic acid solution is added from a pipette. From 0.3 to 0.5 gm of AgIO_3 , measured with sufficient accuracy from a spoon spatula, is added. The tube or flask is stoppered and is shaken *vigorously* for 40 seconds, during which time the proteins are precipitated and the reaction between the AgIO_3 and the chloride is completed.
- (2) *Separation of supernatant solution.* The solution is separated from the solids by centrifugation, by filtering, or by gravity sedimentation, whichever is most convenient.
 - (a) *Centrifugation* for 1 minute at 3,000 rpm suffices. The tube should preferably be capped to prevent evaporation, which might cause measurable increase in concentration if the centrifugation were allowed to over-run the minute. If a 50-ml centrifuge tube is not available, half of the mixture may be centrifuged in a 15-ml tube, since 10 ml of the supernatant suffices for the titration.
 - (b) For *filtration*, a 9-cm, dry, rapidly filtering paper is used.
 - (c) For *sedimentation*, the reaction is carried out by shaking the mixture in a test tube of about 50-ml capacity, and the stoppered tube is then let stand until enough clear supernatant solution has separated to permit withdrawal of 10 ml for titration (sedimentation does not yield enough supernatant for duplicate titrations). From 15 to 30 minutes usually suffices to obtain the

¹⁸ If silver iodate is not available it can be prepared as follows: 23 gm of KIO_3 and 18 gm of AgNO_3 (both of reagent grade, highest purity) are dissolved separately, each in 500 ml of water. The iodate solution is placed in a 2- or 3-liter beaker or jar, and the AgNO_3 solution is added slowly with constant stirring. The precipitate is collected in a Buchner funnel and washed with water, 100 ml at a time. The filtrates are tested by titrating 10 ml portions as described below for the titrations of plasma filtrates, with 0.02308 N thiosulfate. Continue the washing until the volume of thiosulfate used corresponds to no more than the solubility; namely, 0.5 ml of thiosulfate if the temperature is 20°, 1.00 ml if the temperature is 40°, or calculated amounts at intermediate temperatures. The precipitate is dried in a desiccator or an incubator, and is stored in brown glass.

10-ml sample; however, if the tube stands overnight no harm is done. If the supernatant solution is not entirely clear, the tip of the 10-ml pipette used for withdrawing the sample may be wrapped with a wisp of absorbent cotton, which serves as a filter.

(3) *Titration.*

(a) Of the filtrate, 10 ml is pipetted into a 50-ml Erlenmeyer flask. From 0.5 to 1.0 ml of the sodium iodide solution is added (or 0.3 to 0.6 gm of solid NaI). Immediately after adding the iodide the iodine that is set free is titrated with the 0.02308 *N* thiosulfate solution delivered from a 25-ml burette. The thiosulfate solution is delivered in rapid drops (but not in a stream) until the color of the mixture in the flask is only a pale yellow. Two drops of starch solution are then added, and the titration is continued, a drop at a time, until the blue solution suddenly turns colorless. The end-point is so sensitive that it can be located within 0.01 ml of the thiosulfate solution.

(b) The use of starch to sharpen the end-point is desirable, but not necessary, unless picric acid has been substituted for tungstate in the phosphoric-tungstic acid reagent solution. The filtrate obtained with the phosphoric-tungstic acid reagent solution is water clear, and the end-point in it can be obtained without starch by titrating until the last trace of yellow iodine color disappears using a flask with 30 ml of water for color comparison.

(c) When a series of analyses is being done, each 10 ml portion of filtrate is treated with sodium iodide just before it is titrated. If all the filtrates were treated with iodide at the same time, the solutions that would have to wait for some time before titration would lose iodine by volatilization. In 40 minutes the loss is about 1 percent.

(4) *Calculation:*

Millimoles chloride per liter plasma = 10 *T*.

Mg NaCl per 100 ml plasma = 58.5 *T*.

Mg Cl per 100 ml plasma = 35.45 *T*.

T is the number of ml of 0.02308 *N* thiosulfate used in the titration.

e. MICRO ANALYSIS. The analysis can be carried out with 0.2 ml of plasma without changing any of the reagents. The 0.2 ml sample of plasma is mixed in a 15-ml centrifuge tube with 5 ml of the phosphoric-tungstic acid reagent and 100 mg of silver iodate

is added. The tube is closed, shaken for 40 seconds, and centrifuged. The titration is carried out on 2 ml aliquots of the supernatant, with the 0.02308 *N* thiosulfate delivered from a microburette. A burette of 3-ml capacity is desirable, but one of 5 ml is satisfactory.

Calculation:

Millimoles chloride per liter plasma = 50 T.

Mg NaCl per 100 ml plasma = 292.5 T.

Mg Cl per 100 ml plasma = 177.3 T.

T is the number of ml of 0.02308 *N* thiosulfate used in the titration.

f. CONTROL ANALYSIS WITH 0.1 N CHLORIDE STANDARD SOLUTION.

- (1) The entire technic, including the accuracy of the pipettes used and the purity of the sodium tungstate and other reagents, can be checked by analyzing 0.1 *N* HCl, prepared by the constant boiling method. One ml of the 0.1 *N* HCl is measured in the same pipette used for measuring plasma samples and is analyzed for chloride in the same manner. Ten ml of thiosulfate should be required in the titration. If more or less is used, a correction is made in plasma analyses equal to the correction that must be added to or subtracted from the ml of thiosulfate used in this control analysis to bring the thiosulfate to 10 ml. For example, if in control analyses the thiosulfate-used averages 10.04 ml, a correction of 0.04 ml is subtracted from the burette readings in plasma analyses.
- (2) In place of the 0.1 *N* HCl one may use 0.1 molar NaCl or KCl, but must be sure that the salt used is pure and dried at a temperature of 200° or higher.

210. Inorganic Phosphorus¹⁰

a. GENERAL. The term "inorganic phosphorus," in the usage of clinical chemistry is applied to phosphorus in the form of salts of orthophosphoric acid (H_3PO_4). Orthophosphoric acid in solution with molybdic acid forms complex compounds with the latter, of the nature, $\text{H}_3\text{PO}_4 \cdot x\text{MoO}_3 \cdot y\text{H}_2\text{O}$. When the molybdenum is thus combined with the phosphoric acid the hexavalent molybdenum is susceptible to reduction by various reducing agents, with formation of complexes in which the molybdenum is of lower valence than six, and which are intensely blue colored. In the present method excess of molybdic acid is added to combine with all the phosphoric acid, and that portion of the molybdenum which is thus

¹⁰ Fiske and Subbarow, *Journal of Biological Chemistry*, 66, 375 (1925).

combined is reduced by addition of 1,2,4-aminonaphthol-sulfonic acid, with formation of a blue color. The reaction belongs to the class of incompletely defined oxidation-reduction reactions discussed in section VI, chapter 3, and the prescribed conditions must be accurately followed to obtain consistent results.

b. REAGENTS.

- (1) *Molybdic acid solution.* In a 1-liter volumetric flask place 300 ml of water and add gradually 84 ml of concentrated sulfuric acid with constant stirring. Cool the mixture. In a 500-ml Erlenmeyer flask or beaker dissolve 25 gm of ammonium molybdate in 200 ml of water. Rinse the solution into the flask containing the sulfuric acid, mix, and dilute to the 1-liter mark.
- (2) *Trichloroacetic acid, 10 percent solution.*
- (3) *Stock standard phosphate solution.* Dissolve 350.9 mg of pure monopotassium phosphate (KH_2PO_4) in distilled water and make up to 1 liter. Add 10 ml of chloroform as a preservative and keep in a refrigerator. The solution contains 0.08 mg of phosphorous per ml.
- (4) *Dilute working standard solution of phosphate.* Pipette 10 ml of the stock standard into a 100-ml volumetric flask, add 80 ml of the 10 percent trichloroacetic acid, dilute to the mark with distilled water, and mix. Five ml contains 0.04 mg of inorganic phosphorus, equivalent in the analysis to 4 mg of inorganic phosphorus per 100 ml of plasma.
- (5) *Aminonaphthol-sulfonic acid solution.* Dissolve 30 gm of sodium bisulfite (NaHSO_3) and 1 gm of crystalline sodium sulfite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$), or 0.5 gm of anhydrous sodium sulfite, in 200 ml of distilled water. Add 0.5 gm of purified 1, 2, 4-aminonaphthol-sulfonic acid, and stir thoroughly. Store in a dark glass bottle. The reagent should be prepared fresh once a month. The sediment that forms settles to the bottom and need not be filtered out if care is taken not to stir it up when pipetting out portions for analyses.

c. PROCEDURE.

- (1) *Removal of proteins.*
 - (a) Either serum or plasma may be used. There must be no hemolysis, because the cells contain large amounts of organic phosphates which readily hydrolyse with formation of inorganic phosphate. Serum is preferable to plasma because there is usually less danger of hemolysis in the separation of serum from the cells.

- (b) Measure 8 ml of the 10 percent trichloroacetic acid solution into a 25-ml Erlenmeyer flask. While the flask is gently rotated, run in from a pipette 2 ml of serum or plasma. Close the flask with a rubber stopper and shake vigorously a few times. Filter at once through an acid-washed quantitative filter paper, such as Whatman No. 42.
- (2) *Development of color.* Transfer 5 ml of the filtrate to a 10-ml volumetric flask, or, if such a flask is not available, to a test tube marked to contain 10 ml. Into a similar flask or tube measure 5 ml of the working standard solution. To each flask or tube add 1 ml of the molybdic acid solution and 0.4 ml of the aminonaphthol-sulfonic acid solution. Dilute to 10 ml with water, mix thoroughly, and let stand 10 minutes for the color to develop.
- (3) *Colorimetric measurement.* Compare the unknown and the standard. *Calculate:*

$$\text{Mg inorganic P per 100 ml serum} = 4 \times \frac{S}{U}$$

- (4) *Photometric measurement.* Use light of wave length 660 millimicrons. Set the zero point with a blank solution prepared in the same way as the unknown, except that 2 ml of water replaces the 2 ml of serum. *Calculate:*

$$\text{Mg inorganic P per 100 ml serum} = 4 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown (serum) and D_s the density of the standard.

With a given set of reagents the development of color is regular enough to permit one to calculate a k value ($4 \div D_s$) from analysis of a standard solution, and to use this k in subsequent analyses with the same reagents. The calculation then becomes:

$$\text{Mg inorganic P per 100 ml serum} = k D_u$$

If the molybdic acid solution or the aminonaphthol-sulfonic acid reagent is changed, the k should be re-determined.

211. Alkaline and Acid Phosphatase²⁰

a. GENERAL. Blood serum contains enzymes called phosphatases which hydrolyze glycerophosphate, changing its "organic phosphorus" to "inorganic phosphorus." Writing both substrate and

²⁰ Sinowara, Jones, and Rinehart, *Journal of Biological Chemistry*, 142, 921 (1942).

product as free acids, the reaction caused by the enzyme may be indicated as: $C_3H_7O_2.H_2PO_4 + H_2O \rightarrow C_3H_8O_3 + H_3PO_4$. There are two types of serum phosphatase, an "alkaline phosphatase" which acts at pH near 9.3, and an "acid phosphatase" which acts near 5. Each type of phosphatase is estimated by incubating the serum with glycerophosphate for 1 hour in the presence of a buffer that gives the necessary pH, and determining the amount of inorganic phosphorus (of H_3PO_4) liberated by the enzyme. The liberated H_3PO_4 is estimated as the difference by which H_3PO_4 of the incubated mixture exceeds the H_3PO_4 of a control in which action of the enzyme is prevented. The control contains the same mixture as the incubated sample, but in the control the trichloroacetic acid protein precipitant is added to the serum *before* the glycerophosphate, and inactivates the serum enzymes, so that they do not act on the glycerophosphate added later. In the incubated sample the trichloroacetic acid is added *after* the serum and glycerophosphate have been incubated together.

b. APPARATUS. Five or more glass-stoppered, 10-ml graduated measuring cylinders.

c. REAGENTS. The reagents are those used for determination of inorganic phosphorus (par. 210), and in addition the following:

- (1) *Sodium hydroxide, 0.1 N standardized solution.*
- (2) *Acetic acid, 1.0 N standardized solution.*
- (3) *Stock buffered glycerophosphate solution.* Measure 3 ml of petroleum ether and about 80 ml of distilled water into a 100-ml volumetric flask. Add 1 gm of sodium beta-glycerophosphate and 0.85 gm of sodium di-ethyl barbiturate (barbital sodium). Dissolve and add distilled water to bring the volume of the water solution to 100 ml. Mix. Keep in refrigerator.
- (4) *Alkaline glycerophosphate substrate solution.* Into a 100-ml volumetric flask measure 3 ml of petroleum ether, 50 ml of the stock glycerophosphate solution, and 2.8 ml of 0.1 N sodium hydroxide solution. Dilute with distilled water to bring the volume of the *water solution* to 100 ml and mix. The pH should be 10.9, and should be brought to this level if it differs from it by more than 0.1 pH. Keep the solution in a refrigerator. When the solution is mixed with serum in the analysis, the pH of the mixture approximates 9.3.
- (5) *Acid glycerophosphate substrate.* Into a 100 ml volumetric flask measure 3 ml of petroleum ether, 50 ml of the stock buffered glycerophosphate solution, and 5 ml of 1.0 N acetic acid solution. Dilute with distilled water to bring the *water solution* to a volume of 100 ml, and

mix. The pH should be appropriately 5. Keep in refrigerator.

- (6) *Trichloroacetic acid, 30-percent solution.* Dissolve 30 gm of trichloroacetic acid in distilled water and dilute to 100 ml.

d. PROCEDURE FOR ALKALINE PHOSPHATASE.

- (1) *Preparation of incubated sample.* Measure exactly 9 ml of the alkaline glycerophosphate substrate solution into a glass-stoppered cylinder. Place the cylinder in an incubator or water-bath at 38° C. until the solution reaches 38°. Then, from a transfer pipette, add 1 ml of serum, stopper the cylinder, mix, and incubate at 38° for exactly 1 hour, measured from the moment when the serum was added. At the end of the hour add 2 ml of the 30 percent trichloroacetic acid; this stops the enzyme action and precipitates the proteins. Immediately stopper and shake the cylinder, then filter the solution through a low-ash filter paper.
- (2) *Preparation of control sample.* At or near the end of the 1-hour incubation period measure into a glass-stoppered cylinder 9 ml of the alkaline glycerophosphate substrate solution, then 2 ml of the 30 percent trichloroacetic acid, and mix. After the trichloroacetic acid add slowly 1 ml of serum, stopper, shake, and filter.
- (3) *Development of color in the filtrates of both samples and in the standard.* Measure 8 ml of each sample filtrate, representing 8/12 of a ml of serum, into a 10-ml glass-stoppered graduated cylinder. Into a third cylinder measure 3 ml of water and 5 ml of the standard phosphate solution, described for the determination of inorganic phosphorus. (See par. 210.) (The 5 ml of standard solution contains 0.04 mg of phosphorus.) Into each of the 3 cylinders measure 1 ml of the molybdic acid reagent and 0.4 ml of the aminonaphthol-sulfonic acid reagent, followed by distilled water to the 10-ml mark. Mix immediately, and let stand 10 minutes for the color to develop.
- (4) *Measurements, colorimetric or photometric.* The unknowns are compared with the standard exactly as described for inorganic phosphorus. (See par. 210.)
- (5) *Calculations.*
 - (a) *Inorganic phosphorus.* The inorganic phosphorus in mg per 100 ml of serum is calculated exactly as in the determination of inorganic serum phosphorus (par. 210) except that, in place of the factor 4, the factor 6

is here used, because the 8 ml of filtrate used for the analysis in the phosphatase estimation represents only 2/3 of a ml of serum. Hence:

$$\begin{aligned} \text{Mg inorganic phosphorus per 100 ml serum} &= 6 \times \frac{S}{U}, \text{ or} \\ &= 6 \times \frac{D_u}{D_s}. \end{aligned}$$

- (b) *Phosphatase units.* One unit is the amount of phosphatase that will set free 1 mg of inorganic phosphorus from the glycerophosphate in 1 hour's incubation. Hence:

$$\text{Phosphatase units per 100 ml serum} = P_1 - P_2.$$

P_1 is the mg of inorganic phosphorus per 100 ml of serum in the incubated sample, P_2 the mg of inorganic phosphorus per 100 ml serum in the control sample.

e. **PROCEDURE FOR ACID PHOSPHATASE.** Follow the procedure described for alkaline phosphatase in all details except that the *acid glycerophosphate substrate solution* is used instead of the alkaline glycerophosphate.

f. **NOTES.**

- (1) Inhibition of phosphatase activity occurs when the liberated inorganic phosphorus exceeds 60 mg per 100 ml of serum. When such a high value is encountered, dilute the serum or plasma to 2 or 4 volumes with 0.9 percent sodium chloride solution, and repeat the determination. Multiply the result by the dilution factor, 2 or 4.
- (a) If a photometer is used, and the liberated inorganic phosphorus is less than 60 mg, the reading may usually be taken without preliminary dilution.
- (b) If a colorimeter is used, however, accurate reading requires dilution whenever the inorganic phosphorus content of the incubated serum exceeds 12 mg per 100 ml. Do not dilute serum to more than 4 volumes, however, because the buffer effect of the serum might thereby be so diminished that the mixture of the diluted serum with the acid glycerophosphate solution would be more acid, and with the alkaline glycerophosphate more alkaline, than the respective pH values of 5 and 9.3 needed for action of the enzymes.
- (2) Results for alkaline phosphatase by the present method tend to be slightly higher than those yielded by the Bodansky method, in which the pH of the incubated sample is approximately 8.6, instead of the present 9.3.
- (3) In the determination of either acid or alkaline phos-

phatase, the value obtained for the inorganic phosphorus content of the *control* sample is the inorganic phosphorus of the serum itself. Hence this value can be reported along with the phosphatase activity if desired.

212. Calcium of Serum²¹

a. GENERAL. The calcium is precipitated as $\text{Ca}_3(\text{PO}_4)_2$ and the phosphate in the precipitate is determined. Oxalated plasma cannot be used, since the calcium is removed by the oxalate.

b. REAGENTS. In addition to the *reagents required for the inorganic phosphorus* determinations the following are required:

- (1) *Stock standard calcium solution.* Prepare a stock solution by dissolving 0.4991 gm of pure calcium carbonate in about 50 ml of 10 percent trichloroacetic acid in a 1,000-ml volumetric flask (Iceland spar is preferred, if available). Shake well, and when evolution of carbon dioxide has ceased, dilute to the mark with 10 percent trichloroacetic acid.
- (2) *Dilute working standard calcium solution.* Transfer 10 ml of the stock solution to a 100-ml volumetric flask, add 70 ml of 10 percent trichloroacetic acid, and dilute to the mark with distilled water. Five ml equals 0.1 mg of calcium.
- (3) *Alkaline alcohol wash reagent.* In a 100-ml cylinder place 58 ml of 95 percent ethyl alcohol, add 10 ml of amyl alcohol, and make up to 100 ml with distilled water. Add 2 drops of 1 percent phenolphthalein and then 5 percent sodium hydroxide, a drop at a time, with repeated shaking until a distinct pink is obtained.
- (4) *Sodium hydroxide, 25 percent solution.*
- (5) *Trisodium phosphate, Na_3PO_4 5 percent solution.*

c. PROCEDURE.

- (1) Two ml of serum are precipitated with 8 ml of 10 percent trichloroacetic acid in the same manner as in the phosphorus determination. Where phosphorus and calcium are to be determined on the same specimen, sufficient filtrate for both determinations may be obtained by precipitating 3 ml of serum with 12 ml of trichloroacetic acid.
- (2) To a graduated centrifuge tube, transfer 5 ml of the filtrate and to another similar tube transfer 5 ml of the working standard calcium solution. The tips of the centrifuge tubes used must be sufficiently narrow so that the

²¹ Roe and Kahn, *Journal of Biological Chemistry* 67, 585 (1926).

external diameter at the 0.1-ml mark does not exceed 7 millimeters but must not be too finely drawn out. They must be absolutely clean, and when not in use, should be kept immersed in dichromate-sulfuric acid cleaning solution. No reliance should be placed on the graduation marks, as they have been found to be inaccurate in many tubes, and the 10-ml mark should be checked. If found inaccurate, a new mark should be made. To each tube add 1 ml of 25 percent NaOH, mix by twirling, and allow to stand for 5 minutes. Then add 1 ml of the trisodium phosphate solution, and allow the mixture to stand for 1 hour to complete precipitation of the calcium phosphate.

- (3) Centrifugalize for 2 minutes. Decant the supernatant fluid with one smooth movement that will not disturb the precipitate. With the tube still inverted, the mouth is placed upon a clean filter paper and allowed to drain several minutes, after which time any remaining fluid that may have collected on standing is removed from within the mouth of the tube by touching it with a slip of filter paper or with a clean piece of gauze. Add from a pipette about 3 ml of alkaline alcohol wash reagent in such a manner as to break up the mat of $\text{Ca}_3(\text{PO}_4)_2$ in the bottom of the tube. This is done by using a bulb pipette with a fine tip and blowing forcefully, directing the stream upon the calcium phosphate precipitate. If the calcium phosphate mat is not broken up completely by this procedure, it must be fragmented thoroughly with a clean glass stirring rod. The walls of the tube are now washed down with an additional 2ml of the alkaline alcohol wash reagent. The tubes are centrifugalized again for 2 minutes, then decanted and drained as above. Wash the precipitate once more in the same way.
- (4) Redissolve the precipitate in both the standard and unknown tubes in 4 ml of 10 percent trichloroacetic acid; add to each tube 1 ml of the molybdic acid reagent, that is used in the phosphorus determination (par. 210), and 0.4 ml of the aminonaphthol-sulfonic acid reagent, also the same as that for the phosphorus determination. Dilute to the 10-ml mark with distilled water, mix, and allow to stand 10 minutes for the color to develop.

d. COLORIMETRIC MEASUREMENT. The unknown and the working standard are compared.

Calculation:

$$\text{Mg Ca per 100 ml serum} = 10 \times \frac{S}{U}.$$

S is the reading of the standard, U the reading of the unknown.

e. PHOTOMETRIC MEASUREMENT. Prepare a reagent blank as follows: To 4 ml of 10 percent trichloroacetic acid add 1 ml of the molybdic acid solution and 0.4 ml of the aminonaphthol-sulfonic acid solution. Dilute to 10 ml and mix.

Set the zero of the photometer with distilled water. Wave length 660 millimicrons.

With the zero thus set by the water blank, read the optical densities of the reagent blank, the standard, and the unknown.

Calculation:

$$\text{Mg Ca per 100 ml serum} = 10 \times \frac{D_s - D_r}{D_s - D_b}$$

D_s is the optical density of the unknown, D_s the density of the standard, and D_b the density of the reagent blank.

f. CAUTION.

(1) (a) It has been found impossible to obtain reagents which are absolutely free of calcium or of other substances that produce with the reagents the same color as calcium. Therefore the standard solution read in the colorimeter or photometer is prepared, not from a standard phosphate solution, but from the standard calcium solution put through all the processes of precipitation, etc., the same as the serum sample. In this way the effects of color-producing substances in the reagents are balanced, provided the impurities are not too great, nor the difference in calcium content between standard and serum too wide.

(b) The interfering impurities most likely to be encountered in the reagents are calcium and silicate. Silicate forms molybdosilicate complexes, in which, as in molybdophosphate, the Mo^{+++++} is reduced to a lower valence with formation of blue color when treated with aminonaphthol-sulfonic acid. Calcium is likely to contaminate the filter papers. It is necessary to use acid-washed, "ash-free" quantitative filter paper in filtering the plasma protein precipitate. The doubly acid-washed Whatman No. 42 is likely to be calcium-free. Silicate is likely to get into the 25 percent NaOH solution and the 5 percent Na_3PO_4 solution as the result of the solvent effect of these alkaline solutions on their glass containers. These solutions should be kept in pyrex glass containers, or, *still better*, in *paraffin-coated bottles*, described as containers for sodium hydroxide in paragraph 136. If the solutions

are kept in glass bottles it is desirable to use tall ones; insoluble silicates detached from the glass settle as a precipitate to the bottom, and portions of the supernatant solution can be drawn off clear for use in analyses. Since some of the silicate is likely to be in solution, however, this precaution is no guarantee that silicates will not affect the analysis.

- (2) If a specimen must be shipped for analysis, the trichloroacetic acid filtrate should be sent, not the serum itself. Phosphates in the serum may free inorganic PO_4 from the organic phosphates, and thereby precipitate calcium, so that if the serum were shipped not all the calcium might remain in solution.
- (3) Syringes for taking blood specimens should be carefully sterilized in distilled water to avoid contamination with calcium salts.

213. Carbon Dioxide Capacity of Plasma

(Van Slyke and Cullen, *Journal of Biological Chemistry*, 30, 289 (1917))

a. GENERAL. The plasma from oxalated blood is shaken in a separatory funnel filled with an air mixture whose carbon dioxide tension approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of the saturated plasma is then acidified within a suitable pipette, and its carbon dioxide is liberated by the production of a partial vacuum. The liberated carbon dioxide is then placed under atmospheric pressure, its volume accurately measured, and the volume corresponding to 100 ml of plasma calculated.

b. REAGENTS AND APPARATUS.

- (1) The apparatus used is illustrated in figure 18. It is made of strong glass in order to withstand the weight of the mercury without danger of breaking, and is held in a strong clamp, the jaws of which are lined with rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod 6 or 8 millimeters in diameter should be so arranged as to project under the cock *f* between *c* and *d*. Three hooks or rings at the levels 1, 2, and 3 serve to hold the leveling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy-walled rubber tube.

- (2) It is necessary that both stopcocks be properly greased and absolutely airtight; and it is also essential that they (especially *f*) should be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose, but it has been found that fine wire springs, applied in the same manner as the rubber bands, are stronger and more durable.

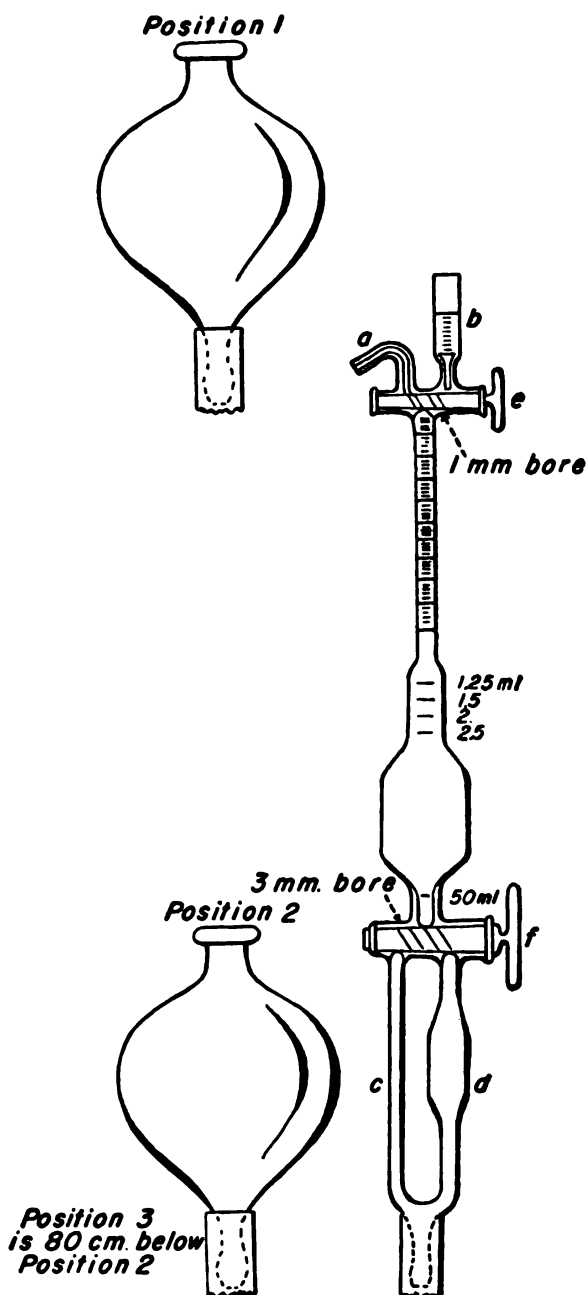


Figure 18. Van Slyke apparatus for determination of carbon dioxide in blood.

- (3) Before the apparatus has been used, or whenever air has been admitted to it, as in renewing mercury or through leaks, the rubber tubing, and even the glass walls may retain a considerable amount of gases which must be evacuated before use. To test the apparatus for tightness and freedom from gases, raise the leveling bulb and completely fill the apparatus with mercury. The bulb is then lowered to position 3, so that a Torricellian vacuum is obtained, the mercury falling to about the middle of *d*; the bulb is then raised again. If the apparatus is tight and gas-free, the mercury will refill it completely and will strike the upper cock with a sharp click. If there is any gas in the apparatus, it will act as a cushion; the click will not be heard and a bubble will remain above the mercury. In this case expel the collected gas through cock *e*, and repeat the procedure until all the gas has been evacuated. After the apparatus has been freed from gases, it can be used repeatedly and indefinitely without further trouble from this source, if no air is admitted and there is no leak. It is always desirable, however, before making the first determination of a series, to test the apparatus as described above.
- (4) After each analysis the apparatus may be used immediately for another determination; the slight amount of carbon dioxide of the old solution that remains on the inner surface of the pipette is negligible. When a series of analyses is finished, the pipette is rinsed several times with water, and is left completely filled with water. If the apparatus is used only occasionally, it is well to lower the mercury below the lower cock, clean both cocks, and place a strip of filter paper between the stopper and the shell of each cock, to insure that the cock will not "freeze" while standing.

c. PROCEDURE.

(1) *Drawing blood and obtaining plasma.*

- (a) It is essential that the blood be collected with minimal gain or loss of carbon dioxide, as increase of CO_2 in the whole blood causes interchange of Cl and HCO_3 ions between cells and plasma in such a way that the CO_2 capacity of the plasma is increased, while loss of CO_2 from the whole blood decreases the plasma CO_2 capacity. Consequently accumulation of CO_2 in the tapped vein is avoided by using a tourniquet, either not at all, or for as short a time as possible. When stasis is used, the ligature is released as soon as the

vein is entered, and a few seconds time is allowed for the stagnant blood to flow out of the vein before the main portion of the blood sample is drawn. It is equally necessary to avoid loss of CO_2 from the whole blood in vitro before centrifugation. During the transfer of the blood sample from the syringe to the centrifuge tube the tip of the syringe needle is placed near the bottom of the centrifuge tube, so that the blood does not fall through the air. The centrifuge tube should contain not less than 1 nor more than 2 mg of oxalate for each ml of blood. The blood is stirred with a rod the least amount necessary to dissolve the oxalate; unnecessary stirring is avoided in order to avoid loss of CO_2 from the blood. The tube with the blood must not be shaken or inverted.

- (b) Centrifugation is begun as quickly as possible, at most not longer than $\frac{1}{2}$ hour after the blood is drawn.
 - (c) The clear plasma is pipetted off, and is either transferred directly to a separatory funnel (fig. 19) for continuation of the analysis, or is stored in a small paraffin-lined test tube for later analysis. In such a tube, stoppered and kept in a refrigerator, the alkali reserve of the plasma will remain unchanged for a week. If the plasma is left in contact with ordinary glass for a few hours enough alkali may dissolve from the glass to increase measurably the CO_2 capacity.
- (2) *Saturation of plasma with alveolar carbon dioxide.* Transfer to the 300-ml separatory funnel shown in figure 19, 3 or 4 ml of the plasma, connect the funnel with the bottle of glass beads (fig. 19), and replace the air in the funnel with alveolar air from the lungs of the analyst. For this purpose, the analyst, without inspiring more deeply than normal, expires as quickly and completely as possible through the bottle of glass beads and the separatory funnel, while they are connected as shown in figure 19. Passage first over the glass beads serves

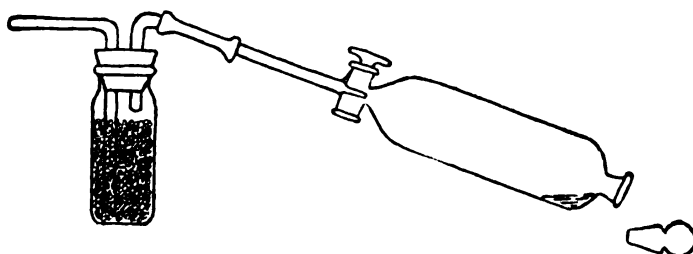


Figure 19. Apparatus for saturating plasma with carbon dioxide.

to condense on them excess moisture from the breath; otherwise it would condense on the walls of the funnel and measurably dilute the plasma.

(3) *Determination of carbon dioxide.*

- (a) Before beginning a determination the apparatus is rinsed and, if necessary, freed of adherent gases, as described in *b* above. The chamber of the apparatus and the 2 capillaries above cock *e* are filled with mercury.
- (b) A drop of caprylic alcohol is placed in the empty cup, and is drawn down into the capillary, but not so far as to leave an air space in the capillary below the cup. Then 1 ml of water is placed in the cup, and 1 ml of plasma is run slowly under the water, with care to deposit the plasma in a layer below the water, and not to mix the two fluids. The plasma is then slowly admitted to the chamber, followed by the 1 ml of water, which washes with it the portions of plasma adherent to the wall of the cup.
- (c) Finally about 1 ml of the 1*N* sulfuric acid is placed in the cup, and enough is admitted into the chamber to make the total aqueous fluid there reach exactly to the 2.5-ml mark. During admission of these fluids no air must be admitted into the chamber.
- (d) After the acid has been admitted, close cock *e* and place several drops of mercury in the cup under the acid that remains there. Let a little of the mercury run through the cock in order to seal the latter and make it hold a complete vacuum. Leave enough mercury above the cock to fill the capillary. Whatever sulfuric acid solution remains in the cup is removed, most conveniently by suction.
- (e) If the amount of plasma available is small, a little more than 0.5 ml is saturated with carbon dioxide in a 50 ml funnel, and exactly 0.5 ml used for the determination. In this case the quantities of water and acid used to wash the plasma into the apparatus are halved, so that the total volume of water solution introduced is only 1.25 ml and the observed volume of gas is multiplied by 2 before proceeding with the calculation.
- (f) The mercury bulb is now lowered and hung at position 3 and the mercury in the pipette allowed to run down to the 50-ml mark, producing a Torricellian vacuum in the apparatus. When the mercury meniscus has fallen to the 50-ml mark, the lower cock *f* is closed, the level-

ing bulb is replaced in position 2, and the pipette removed from the stand. Equilibrium of the carbon dioxide between the 2.5 ml of water solution and the 47.5 ml of free space is obtained by turning the pipette upside down 15 or more times, thus thoroughly agitating the contents. The pipette is then replaced in the clamp and the leveling bulb is lowered again to position 3.

- (g) By opening the cock *f*, the water solution is allowed to flow from the pipette completely into the chamber *d* without, however, allowing any of the gas to follow it. The leveling bulb is then raised in the left hand, while with the right the cock *f* is turned so as to connect the pipette with *c*. The mercury flowing in from *c* fills the body of the pipette and as much of the calibrated stem at the top as is not occupied by the gas extracted from the solution. A few hundredths of a milliliter of water, which could not be completely drained into *d*, floats on top of the mercury in the pipette; the error caused by the reabsorption of carbon dioxide into this small volume of water is negligible if the reading is made at once. The mercury bulb is held at such a level that the gas in the pipette is under atmospheric pressure, that is, level with the top of the mercury column in the pipette stem, and the volume of gas is read on the scale.
- (h) In order to have the column of mercury and water solution in the pipette exactly balanced by the mercury outside, the surface of the mercury in the leveling bulb should be raised until it is level with the mercury meniscus in the pipette and then, for entire accuracy, raised above the latter meniscus by a distance equal to one-thirteenth of the height of the column of water above the mercury in the pipette. As the water column should never be more than 10 millimeters high, the correction that has to be estimated is less than 1 millimeter of mercury, not enough to influence results appreciably.

d. CALCULATION.

- (1) Multiply the observed volume of gas by the fraction,

$$\frac{\text{mm barometric pressure}}{760}.$$
- (2) Note in the first or sixth column (table XVII), the number that is nearest to the product thus obtained.

- (3) In the row of figures to the right of that number note the figure that is under the temperature value nearest to the room temperature at which the analysis was done. The figure thus noted is the answer, in terms of bicarbonate CO_2 , or the CO_2 capacity.

Example: The volume of gas measured is 0.71 ml. The temperature is 22° , the barometer 750. Multiply 0.71×750

— and obtain 0.70. Opposite 0.70 in the table, in the 760

column under 20° , read the result, 57.6 volumes percent of CO_2 .

Table XVII is computed with corrections for temperature effect on gas volume, for physically dissolved CO_2 and air, and for CO_2 solubility. The CO_2 capacity obtained after making these corrections is practically the bicarbonate CO_2 . About 0.2 percent of the plasma CO_2 is in the form of CO_3^{--} , and a slight amount is bound to the plasma proteins as carbamate, but these amounts are negligible compared with HCO_3^{--} .

214. Carbon Monoxide in Blood

(Van Slyke and Salvesen, J. Biol. Chem., 40, 103 (1919) modified by Van Slyke and Plazin (Peters and Van Slyke, Quantitative Clinical Chemistry, Methods, 1943 Edition, p. 261))

a. GENERAL.

- (1) The analysis is carried out with the same apparatus used for plasma CO_2 capacity (par. 213), and employs 3 ml samples of blood. The determination requires about 10 minutes. The reagents last indefinitely as prepared, and can therefore be kept ready for emergency analyses.
- (2) Blood containing CO contains also CO_2 , O_2 , and N_2 gases. The object of the analysis is to get the CO, in gas form, separated from the other three gases and measure it. The O_2 is removed at the start by combining it with hydrosulfite. The N_2 is then extracted from the blood and ejected from the apparatus. The CO and CO_2 remaining in the blood solution are then set free by acid ferricyanide, which dissociates the HbCO into methemoglobin and free CO. From the mixture of CO and CO_2 gases, the CO_2 is absorbed by a few drops of NaOH solution, and the remaining CO gas is measured.

b. APPARATUS.

- (1) The apparatus is shown in figure 18. The handling and cleaning of the apparatus are described in paragraph 213. Before using the apparatus for carbon monoxide determination it is important to test it for tightness, as described in paragraph 213.
- (2) In addition to the gas apparatus, a *glass spoon* is used for measuring 30 to 40 mg of sodium hydrosulfite powder. A glass tube of 4-mm bore and about 15 cm long is melted together near one end and is bent at a right angle at the melted point. The short end is cut off to form a cup 3 to 4 mm deep, to which the rest of the tube serves as handle. After once weighing 35 mg of hydrosulfite into the cup one can subsequently measure the amount by eye within ± 5 mg.

c. REAGENTS.

- (1) *Caprylic alcohol*, to prevent foaming.
- (2) *Sodium hydrosulfite* (also called hyposulfite) ($\text{Na}_2\text{S}_2\text{O}_4$) powdered.
- (3) *Saponin = borate solution*. Dissolve 3 gm of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) and 1 gm of saponin in 100 ml of water. The saponin serves to dissolve the red blood cells and make them accessible to the reagents. The borax provides the alkalinity necessary to prevent loss of carbon monoxide from the HbCO during preliminary extraction of the N_2 gas from the blood.
- (4) *Potassium ferricyanide solution*. Dissolve 32 gm of $\text{K}_3\text{Fe}(\text{CN})_4$ in warm water and dilute to 100 ml.
- (5) *Acetate buffer of pH approximately 6*. Dissolve 75 gm of sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) in 100 ml of water. To the solution add 15 ml of glacial acetic acid.
- (6) *Sodium hydroxide solution, 1N* (approximate).

d. PROCEDURE.

- (1) Begin the analysis with the chamber of the apparatus (fig. 18) and the two capillaries above cock *e* full of mercury. The leveling bulb is in position 2, as shown in figure 18, and remains in this position throughout the analysis, except when momentarily lowered or raised.
- (2) Drop 4 drops of caprylic alcohol into the cup of the apparatus, and run the alcohol down into the chamber until only enough remains above the latter to fill the capillary at the bottom of the cup. When admitting liquids into the chamber leave cock *e* open to the cup, and regulate the flow with cock *f* at the bottom of the chamber.

- (3) Pipette 3 ml of blood into the cup, run the blood down into the chamber, *slowly* to obtain good drainage, again leaving enough of the fluid above the chamber to fill the capillary below the cup.
- (4) Fill the cup to its 5 ml mark with the saponin-borate solution.
- (5) From the glass spoon drop 30 to 40 mg of pulverized $\text{Na}_2\text{S}_2\text{O}_4$ into the saponin-borate solution in the cup, and dissolve the hydrosulfite quickly by stirring a few times with a slender rod. The stirring also detaches from the wall of the cup the film of blood left after running the main part of the blood sample into the chamber. At once, before air can oxidize the hydrosulfite, run the solution into the chamber, again leaving enough above the cock to fill the capillary. A drop of mercury is placed in the cup and used to fill the capillary bore of cock *e*, and the cock is closed.
- (6) To extract the N_2 gas from the blood solution, the leveling bulb is lowered for a moment to position 3, and mercury is withdrawn from the chamber, leaving in the chamber all the blood solution and an evacuated, gas-free space above the solution. Cock *f* at the bottom of the apparatus is closed, and the leveling bulb is returned to position 2. Remove the chamber from the clamp. Hold the upper part in the left hand, and with the right hand give the lower part a rapid back-and-forth motion causing the blood to rotate about the side wall of the chamber. Continue the rotation for 2 minutes by the watch, to extract all the N_2 gas from the solution. The cock at the bottom is then turned to permit mercury to return to the chamber, and the leveling bulb is raised to position 1.
- (7) The bubble of N_2 is ejected from the chamber. To do this leave the leveling bulb high in position 1, close the lower cock of the apparatus and open the upper cock. Then slowly reopen the lower cock and admit mercury through it into the chamber until the bubble at the top is ejected. Just enough solution to fill the capillary below cup *b* is permitted to flow up after the bubble.
- (8) The carbon monoxide gas is now set free from the HbCO by the action of acid ferricyanide. Into cup *b* measure 1.5 ml of the 32 percent ferricyanide solution and 0.5 ml of the acetate buffer solution. The solutions are mixed with a rod, and 1.5 ml of the mixture is drawn down into the chamber. The acid ferricyanide dissociates the HbCO into methemoglobin and free CO . Cock *e* is closed

and sealed with mercury, the mercury in the chamber is again withdrawn from the bottom, and the blood is again whirled about the walls of the chamber as in the extraction of the N_2 . The CO and most of the CO_2 of the blood pass out of the liquid phase into the gas space in the chamber.

- (9) After the 2-minute extraction is completed, the leveling bulb is lowered to position 3, and as nearly as possible all of the blood solution but none of the gas is drawn into the bulb *d* below the chamber. Then the leveling bulb is returned to medium position 2, and mercury is re-admitted into the chamber, *this time through tube c*, leaving the blood solution trapped in the bulb below the chamber. The chamber is left connected with tube *c*.
- (10) From the bubble of mixed CO and CO_2 , that collects above the mercury at the top of the chamber, the CO_2 is now absorbed with the 1 *N* sodium hydroxide solution. About 1 ml of the 1 *N* NaOH is placed in cup *b*, and a drop at a time is permitted to flow down the side of the graduated tube at the top of the chamber. As the alkali solution enters, the bubble contracts because of absorption of its CO_2 . After admission of a few drops of alkali no more contraction occurs, and absorption of CO_2 is complete. If, during admission of the alkali, it should run down the graduated tube in a solid column instead of trickling down the side, drop a little mercury into cup *b* and let it run in minute droplets through the cock into the chamber. The mercury will break the column of the solution, and cause absorption of the CO_2 . About 0.1 ml of the NaOH solution suffices to absorb all the CO_2 . When absorption is complete a little mercury is run through cock *e* to detach the droplet of alkali solution that adheres below the cock. The bore of the cock is left full of mercury.
- (11) The CO bubble is now measured. Leaving the chamber connected with tube *c*, the leveling bulb is raised from position 2 until the mercury surface in the bulb is above the mercury surface in the chamber by a height equal to one-thirteenth the height of the small column of alkali solution over the mercury in the chamber. In this manner the column of solution is balanced, and the gas in the chamber is put under atmospheric pressure. The height by which the mercury surface in the bulb should be held above that in the chamber can be estimated with sufficient accuracy by the eye, as it is only 2 or 3 mm.

Table XVII. Table for Calculation of Carbon Dioxide Combining Power of Plasma (Van Slyke and Cullen)

Observed volume gas B $\times \frac{\text{---}}{760}$	Ml of CO ₂ reduced to 0° C., 760 mm, bound as bicarbonate by 100 ml of plasma				Observed volume gas B $\times \frac{\text{---}}{760}$	Ml of CO ₂ reduced to 0° C., 760 mm, bound as bicarbonate by 100 ml of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.8	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

(12) The room temperature near the apparatus is taken, and if an exact result is desired the barometer is read.

(13) To clean the apparatus after an analysis let 2 or 3 ml of the 1 N sodium hydroxide solution mix with the blood

Table XVIII. Factor F for Reducing Volume of Moist Gas at Atmospheric Pressure and Temperature to Volume Occupied by Dry Gas at 0°C. and 760-mm Pressure

Temperature at which volume of moist gas is measured	Observed barometer reading, uncorrected for temperature				
	700	720	740	760	780
	Factors				
10	0.874	899	925	950	975
12	866	891	916	942	967
14	858	883	908	933	958
16	851	976	900	925	950
18	842	867	892	916	941
20	834	858	883	907	932
22	825	849	874	898	922
24	816	840	864	888	912
26	807	831	855	879	903
28	797	821	845	869	892
30	788	812	835	859	882
32	778	802	825	849	873
34	768	792	815	839	862
36	758	781	804	828	851
38	747	770	793	816	839

solution in the chamber; this dissolves clots of methemoglobin that have formed from the action of the acid ferricyanide. The dark, alkaline blood solution is ejected from the chamber, and the chamber and the bulb beneath it are rinsed with water until all the colored material is removed. If clots of methemoglobin still adhere to the walls, they can be dissolved in water to which a little sodium hydrosulfite and a few drops of 1 *N* NaOH are added.

e. CALCULATION. From the observed gas volume, 0.025 ml is subtracted to correct for that volume of air introduced with the ferricyanide solution and measured with the CO gas. The remaining gas volume (CO) is reduced to the volume it would occupy at 0°, 760 millimeter pressure, and is multiplied by 33.3 and the factor F to obtain, from the observed CO volume extracted from 3 ml of blood, the volume, reduced to standard conditions, contained in 100 ml, or the "volumes percent" of the gas in the blood.

$$\text{Volumes per cent CO in blood} = 33.3 (V - 0.025) \times F.$$

V is the volume of gas in ml measured after absorption of the CO₂, and F is the factor from table XVIII for reducing gas volume to those occupied at 0°, 760 mm.

f. PRECAUTIONS. Since the CO gas is measured as the residual gas left after removal of all other gases, any air leaking into the apparatus during the analysis would be measured as CO. Hence it is important to note the remarks in paragraph 213 *b* on "testing the apparatus for tightness and freedom from gases."

215. Copper Sulfate Gravity Method for Plasma or Serum Protein, Blood Hemoglobin, and Hematocrit

(Phillips, Van Slyke, Dole, Emerson, Hamilton and Archibald. BuMed News Letter, June (1943) and in Bull. U. S. Army Med. Det., 71, 66 (1943))

a. GENERAL.

- (1) Drops of blood or plasma are delivered from a syringe needle, medicine dropper, or glass capillary into copper sulfate solutions of known specific gravity. Each drop, on entering the solution becomes encased in a sack of precipitated copper proteinate, and remains as a discrete drop without change of gravity for 15 or 20 seconds, during which its rise or fall reveals its gravity relative to that of the solution. The size of the drop is not a factor. No temperature corrections are needed because the temperature coefficients of expansion of the copper sulfate solutions are almost exactly the same as those of blood or plasma of equal specific gravity. The copper sulfate solution cleans itself after each determination because, within a minute or two after the test, the material of the drop settles to the bottom as a precipitate. The standard copper sulfate solutions are prepared by dilution from a stock solution that has, at 25° C., 1.1000 times the density of water at that temperature. This stock solution may either be prepared by weight from pure $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, or by dilution from a saturated solution.
- (2) From the specific gravity of plasma or serum the protein concentration can be estimated with an accuracy that is usually within 0.3 gm per 100 ml. From the difference between whole blood gravity and plasma gravity, the hemoglobin can be calculated, usually within an accuracy of 0.3 gm per 100 ml of blood, and the hematocrit can be estimated. From the specific gravity of whole blood alone a somewhat less precise but usually reliable estimate of the hemoglobin can be made. The calculations are made by a line-chart. Blood and plasma gravities can be meas-

ured, and the hemoglobin, hemotocrit, and plasma proteins calculated, in about two minutes.

- (3) For precise work, namely, gravities accurate within ± 0.0002 , a "laboratory set" of 60 copper sulfate solutions graded at intervals of 0.001 in specific gravity is used; 20 solutions cover the plasma range, 1.016–1.035, and 40 cover the range for whole blood, 1.036–1.075. For rougher work, with gravities accurate to ± 0.001 , a "field set" of 16 solutions with gravities at intervals of 0.004 suffices to cover the entire range of blood and plasma. Approximate field determinations of whole blood gravities can be done with a pocket set of 6 solutions.
- (4) If it is necessary only to find whether the specific gravity of blood is above or below a given level, as in examination of blood donors, a single copper sulfate solution suffices.

b. APPARATUS.

- (1) *Bottles or tubes for standard copper sulfate solutions.*
One or more of the following sets will be needed:
 - (a) *Laboratory set for 100 ml standards covering the range of blood and plasma.* Sixty cylindrical "prescription bottles" of about 120 ml (4 ounces) capacity cover the range of both blood and plasma, 1.075–1.016. The stoppers may be, in the order of preference, first screw-cap, then rubber, cork, or glass. The 100 ml of copper sulfate solution held by each of these bottles can receive 100 drops of blood or plasma before the solution must be renewed.
 - (b) *Portable laboratory set covering same range.* Sixty 30 ml (1 ounce) bottles can be packed in a space 10 inches square and 4 inches deep. The 25 ml portions of solution held by these bottles suffice for analyses of 25 bloods and their plasmas.

For ward use it may be convenient to use a set of standards in *small test tubes or vials*, such as Kahn antigen test-tubes, with 5 ml of copper sulfate solution in each tube. Each tube can be used for 5 drops of plasma or blood. After such a set has been used, all solutions that have received any blood or plasma are replaced by fresh solution before the set is used again. When standing with solutions in them, these tubes must be stoppered to prevent change of gravity by evaporation, which would affect the small volumes in the tubes more rapidly than the larger volumes in bottles.

- (c) *Field set for approximate analyses covering range of whole blood and plasma.* This includes 16 screw-cap bottles to hold solutions covering the range 1.016–1.074 at intervals of 0.004. It serves for gravities accurate within ± 0.001 . The bottles may be 30, 60, or 120 ml capacity, according to the number of bloods to be analyzed, and to the space and weight desirable for the set.
- (d) *Pocket field set for whole blood.* This consists of six 30 ml screw-cap bottles to hold solutions of gravities 1.040, 1.046, 1.052, 1.064, 1.070. It serves to estimate whole blood gravities within ± 0.002 .
- (2) *Apparatus for preparing stock copper sulfate solution of gravity 1.100 from weighed portions of 170 or 42.5 grams of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.*
 - (a) For the 170 gram portions, a 1-liter volumetric flask, a 10-ml graduated pipette, and a stoppered bottle capable of holding somewhat more than the volume of the stock solution (one or more liters) to be prepared.
 - (b) For the 42.5 gm portions, a 250-ml volumetric flask, a 5-ml graduated pipette, and a stoppered bottle of 300 ml or more capacity.
- (3) *Apparatus for preparing a saturated copper sulfate solution and therefrom the stock solution.*
 - (a) Three 4-liter bottles.
 - (b) One 1-liter volumetric flask.
 - (c) One 500-ml graduated cylinder.
 - (d) One glass funnel, 18 to 20 cm (7–8 inches) diameter.
 - (e) Cotton, or a loose texture filter paper, preferably folded, to fit the funnel.
 - (f) A precision thermometer, Centigrade or Fahrenheit, for liquids at room temperature.
- (4) *Apparatus for preparing standard solutions from the stock solutions.* To make standard solutions of 100 ml volume, a 100-ml burette and a 100-ml volumetric flask are required. If the standard solutions are to be made in 50 or 25 ml portions, the flask and burette are of 50 or 25 ml capacity.
- (5) *Apparatus for drawing and preparing blood.*
 - (a) *For venous blood.*
 - 1. Glass syringes, 5 or 10 ml capacity.
 - 2. Hypodermic needles, No. 20.
 - 3. Rubber tourniquet.
 - 4. Medicine droppers.

5. *Centrifuge* (can be omitted if only whole blood is to be tested, or if one can wait for blood to clot or sediment till a few drops of serum or plasma can be obtained).

6. *Oxalated test tubes or centrifuge tubes.* Test-tubes of heavy-walled pyrex glass of about 10 ml capacity (125 by 16 mm) are somewhat more convenient than 15 ml centrifuge tubes. The tubes are prepared in advance for 5 ml portions of blood by pipetting into each tube 0.25 ml of Heller and Paul's oxalate solution. (See *c* below.) The solution is spread in a film over the lower half of the tube, and is dried in an incubator, or in air warmed to not over 60° C. or in a vacuum desiccator. Each tube, thus charged with 5 mg of oxalate, is marked on the outside to hold 5 ml.

(b) *For capillary blood.*

1: *Sterile lancets.*

2. *Glass capillary tubes to collect blood.* "Vaccine" capillary tubes, of about 1-mm bore, with rubber bulbs to expel drops of blood, are convenient. The tubes may be 70 or 80 millimeters long if only one drop of blood is to be used, as in examining blood donors, but should be about 150 millimeters long if 3 or 4 drops are to be used, for an approximate hemoglobin estimation. The bores of the tubes must be absolutely clean, or blood will not be drawn into them by capillary attraction. To clean the tubes cover them in a long test tube with chromic acid cleaning mixture for 1 hour or more. Then wash them many times with distilled water, and dry them in an oven. The rubber bulbs mentioned are not entirely necessary, as pressure of the breath can be used to expel a drop of blood from a capillary tube.

c. REAGENTS.

(1) *Heller and Paul's oxalate mixture.* Dissolve 3 gm ammonium oxalate and 2 gm potassium oxalate in 250 ml of distilled water to make a solution containing 20 mg of the mixed oxalate per ml.

(2) *Crystalline copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).*

(a) This is preferably purchased in the form of "fine crystals." Otherwise it must be pulverized before using. Two kilograms, or 4 pounds, will probably suffice an active laboratory for a year.

- (b) For preparation of solutions by weight the sulfate must be the exact composition, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, with theoretical H_2O content. The H_2O is determined by heating 2 or 3 grams of the sulfate in open pyrex weighing bottles at 300° to 350° C. until the weight is constant, which requires 2 or 3 hours. The loss in weight should be 36.06 ± 0.2 percent. Crystals of "U.S.P." sulfate do not usually meet this requirement. "Analytical reagent" grade usually has the correct composition.
 - (c) For preparation of saturated solutions, copper sulfate crystals of commercial or U.S.P. grade are sufficiently pure.
- (3) *Copper sulfate, saturated solution.*
- (a) *Precision of saturation technic.* If water is saturated with copper sulfate at a known temperature the concentration of the solution can be defined with an accuracy which depends on the temperature control. One degree Centigrade rise in temperature increases the solubility about 2 percent. If, as directed below, the temperature of saturation is measured to the nearest 0.5° C., that is to within $\pm 0.25^\circ$ of the exact temperature, the concentration of the saturated solution is exact enough to provide standards for plasma gravities accurate within ± 0.0002 and whole blood gravities within ± 0.0003 , which are about the limits of accuracy of the blood and plasma gravity observations.
 - (b) *Preparation of saturated solution.* The procedure described below provides enough saturated solution to yield more than 4 liters of the stock solution of gravity 1.100, and thence a set of 60 standard solutions of 100 ml each, with enough surplus to provide replacements for the standard solutions which are most used. Smaller or larger amounts of the saturated solution can be prepared by using proportional amounts of copper sulfate and water.
1. Before starting be prepared to carry the preparation through both the saturated solution and the stock solution stages. It is desirable to have an assistant. Make sure that the necessary apparatus (three 4-liter bottles, etc., listed above under "Apparatus (3)") is at hand, clean, and dry. Insert into one of the 4-liter bottles the glass funnel, provided with a wad of cotton, or with a dry, rapidly filtering paper large enough to fill the funnel. Have ready 4

or 5 liters of distilled water or rain water at room temperature.

2. Place 4 pounds (about 1,800 grams) of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in the form of "fine crystals," or of a powder fine enough to go through a 20-mesh sieve, in one of the 4-liter bottles. Measure into the bottle 2,500 ml of distilled water or rain water at room temperature. Stopper the bottle and shake vigorously by repeated inversion, so that the crystals are forced rapidly from one end of the bottle to the other. Continue shaking for 5 minutes by the watch. At the first sign of tiring, pass the bottle to the assistant to carry on the shaking. *As soon as the 5-minute shaking is finished, set the bottle down, immediately insert the thermometer into the solution, and record its temperature to the nearest half-degree Centigrade or degree Fahrenheit.* Then at once decant the solution from the bottle into the prepared funnel, leaving the bulk of the crystals behind in the bottle. Complete the filtration as quickly as possible, keeping the funnel filled with the solution until all the liquid above the crystals has been emptied out of the saturation bottle.
 3. The saturated solution is at once used to prepare the stock solution.
- (c) *Points in preparing the saturated solution.*
1. If saturation is to be attained in 5 minutes the copper sulfate must be *finely divided*, either in the form sold as "fine crystals," smaller than grains of wheat, or else pulverized fine enough to pass through a 20-mesh sieve.
 2. To attain the saturation in 5 minutes it is also necessary that *a sufficient excess of the crystals* be present. Four pounds (1,800 gm) of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to 2.5 liters of water provides this excess; less than this amount may not suffice to produce complete saturation in 5 minutes, even though some of the crystals remain undissolved.
 3. The *temperature* of the saturated solution must be taken at the moment the saturation is completed. *Immediately* at the end of the 5-minute shaking period, insert a thermometer bulb into the solution and hold it there until the mercury thread stops moving (20 to 30 seconds), then record the temperature. The process of dissolving the sulfate cools

the solution to about 3° C. below the temperature that the water had before it was poured upon the crystals. It is at this lower temperature, possessed by the mixture at the end of the saturation process, that the solution is saturated. Table XX is based on the temperature of the supernatant solution taken immediately at the end of the 5-minute shaking, and not on its temperature even a minute or two later, and particularly not after the solution has been decanted, nor subsequently after filtration. By the time the solution has been filtered it will usually be back at room temperature, and an error would be caused if this were recorded as the temperature of saturation. For example, of a solution saturated at 20° (end of 5-minute shaking) table XX indicates that 489 ml are required to contain the 159.5 gm of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to make 1 liter of the stock solution. If the temperature of the saturation were erroneously recorded as 23°, one would, following the table, take only 466 ml to prepare a liter of the stock solution, and its gravity would be 1.0953 instead of 1.1000.

(4) *Stock copper sulfate solution of gravity. $D_{25} = 1.1000$.*

(a) *Composition of solution, and choice of procedures for its preparation.*

1. The stock solution can be prepared in three ways: By dissolving 159.5 gm of pure crystals of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water and making up to 1 liter at 25°; by mixing a weighed amount of crystals with a measured volume of water, sufficient to provide 5.897 gm of water for each gm of crystals; and by saturating water with crystals at a known temperature and diluting a measured volume of the saturated solution to a liter.
2. For preparation from weighed amounts of crystals procedure 2 is usually more convenient than procedure 1, because procedure 2 permits one to prepare in one operation any amount of the solution desired, directly in its permanent container, and to make easily very exact allowance for temperature effects on the volume of water. If weighed portions of pure $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ are available, their use in this manner is simpler also than the saturation technic of procedure 3.

3. On the other hand, the saturated solution used in procedure 3 has the advantages that it can be prepared without either accurately weighed or highly purified crystals; the crystal water content need not be exact, and roughly estimated amounts of commercial or U.S.P crystals can be used to make the saturated solution; hence the saturation technic enables one to apply the method to blood analyses with minimal equipment wherever copper sulfate is obtainable.

Note. Procedures 2 and 3 are accordingly detailed below.

- (b) *Preparation of stock solution from weighed portions of pure $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.* The $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and water are mixed in the ratio of 170 gm of crystals to 1002.4 gm of water. Sealed bottles containing 170 ± 0.2 gm of "fine crystals" of analyzed purity are furnished. For smaller volumes 42.5 ± 0.05 gm of copper sulfate may be used. These weights are convenient because the corresponding amounts of water can be measured in 1 liter or 250-ml flasks, respectively, with slight additional amounts of water varying according to temperature, added from a pipette or burette.

Table XIX. *Volume of Water to Add to Weighed Amount of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to Prepare Stock Solution of Gravity 1.1000*

Temperature of water		ml of water to 170 gm of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	ml of water to 42.50 gm of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
$^{\circ}\text{C}$	$^{\circ}\text{F}$		
10	50.0	1003.6	250.9
12	53.6	1003.8	250.9
14	57.2	1004.0	251.0
16	60.8	1004.3	251.1
18	64.4	1004.7	251.2
20	68.0	1005.1	251.3
22	71.6	1005.5	251.4
24	75.2	1006.0	251.5
26	78.8	1006.5	251.6
28	82.4	1007.0	251.8
30	86.0	1007.7	251.9
32	89.6	1008.3	252.1
34	93.2	1008.9	252.2
36	96.8	1009.6	252.4
38	100.4	1010.4	252.6
40	104.0	1011.2	252.8

Note. The solution contains 1002.4 gm of water per 170 gm $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The volumes of water given in the table are 0.8 ml per liter more than theoretical in order to allow for adherence of this amount to the inside of the flask after 2-minute drainage.

Table XX. Volume of Saturated Copper Sulfate Solution to be Diluted to 1 Liter to Give a Stock Solution of Specific Gravity 1.1000

Temperature of the saturated solution at the time of saturation		Volume of solution to dilute to 1 liter	Temperature of the saturated solution at the time of saturation		Volume of solution to dilute to 1 liter	Temperature of the saturated solution at the time of saturation		Volume of solution to dilute to 1 liter
°C	°F	ml	°C	°F	ml	°C	°F	ml
10.0	50.0	587	20.0	68.0	489	30.0	86.0	424
10.5	50.9	581	20.5	68.9	485	30.5	86.9	421
11.0	51.8	575	21.0	69.8	481	31.0	87.6	418
11.5	52.7	569	21.5	70.7	477	31.5	88.7	415
12.0	53.6	563	22.0	71.6	474	32.0	89.6	412
12.5	54.5	557	22.5	72.5	470	32.5	90.5	410
13.0	55.4	552	23.0	73.4	466	33.0	91.4	407
13.5	56.3	546	23.5	74.3	463	33.5	92.3	404
14.0	57.2	541	24.0	75.2	459	34.0	93.2	401
14.5	58.1	536	24.5	76.1	456	34.5	94.1	398
15.0	59.0	531	25.0	77.0	453	35.0	95.0	395
15.5	59.9	527	25.5	77.9	450	35.5	95.9	392
16.0	60.8	522	26.0	78.8	446	36.0	96.8	389
16.5	61.7	518	26.5	79.7	443	36.5	97.7	387
17.0	62.6	514	27.0	80.6	440	37.0	98.6	384
17.5	63.5	509	27.5	81.5	438	37.5	99.5	381
18.0	64.4	505	28.0	82.4	435	38.0	100.4	378
18.5	65.3	501	28.5	83.3	432	38.5	101.3	374
19.0	66.2	497	29.0	84.2	429	39.0	102.2	371
19.5	67.1	493	29.5	85.1	427	39.5	103.1	368
20.0	68.0	489	30.0	86.0	424	40.0	104.0	365

In a bottle, of size to hold somewhat more than the volume of stock solutions to be made, place one or more of the weighed portions of 170 gm of the sulfate; for example, to make 3,200 ml of stock solution, enough for a complete laboratory set of 100 ml standard solutions, place 3 of the 170 gm portions of sulfate in a 4-liter bottle. Make sure that every particle of the sulfate is transferred to the bottle. Fill a 1-liter flask to the mark with distilled or rain water. Take the temperature of the water. Then add from a 10-ml graduated pipette or a burette enough additional water to the liter in the flask to bring the volume up to that indicated in table XIX for addition to 170 gm of sulfate. Empty the water from the flask into the bottle containing the sulfate. Let the water from the upturned flask drain into the bottle 2 minutes by a watch. In

this manner measure into the bottle one portion of water from the 1-liter flask for each 170-gm portion of copper sulfate.²²

(c) *Preparation of stock solution from a saturated solution.*

1. As soon as the saturated solution is prepared and filtered, measure into a 500-ml cylinder the volume of saturated solution indicated by table XX according to the temperature *previously recorded in the copper sulfate solution at the moment the saturation process was ended*. If the volume required exceeds 500 ml (temperature of saturation below 19° C.) fill the cylinder to the 500 ml mark and add the extra amount, from a small cylinder or a burette, to the solution in the 500-ml cylinder. Pour the entire solution thus measured in the 500-ml cylinder from the latter into the 1-liter volumetric flask. Let the up-turned cylinder drain for 2 minutes into the flask to complete the transfer. Then fill the flask to the mark with distilled water or rain water, stopper, and invert 10 times to mix the solution; then stand the flask upright. The mixing results in a shrinkage of volume, so that the meniscus falls below the mark. To correct for this shrinkage, let the flask stand for 1 minute until the solution drains down from the neck, and then add enough water to bring the meniscus back to the mark. Stopper the flask, again mix the solution, and transfer to the empty 4-liter bottle for storage.
2. Rinse out the liter flask with water, and discard the rinsings. Do not rinse the 500-ml cylinder; measure into it the same volume of saturated copper sulfate solution as before, and decant again into the rinsed 1-liter flask. Fill the flask, mix, adjust for concen-

²² The figures in tables XIX and XX provide a stock solution that has 1.1000 times the density of water when both densities are measured at 25° C. This stock solution at temperatures below 25° has slightly more, at above 25° slightly less, than 1.1000 times the density of water at the same temperatures, because the copper sulfate solution has a slightly greater coefficient of expansion than water. Directions in earlier publications of this method yielded a stock solution that had, at whatever temperature the solution was prepared, 1.1000 times the density of water at that temperature. The coefficients of expansion of blood, plasma, and copper sulfate solutions are so related that the present stock solution provides standard solutions which give slightly more accurate plasma protein and blood hemoglobin values at temperatures outside the range 20° to 30° inclusive. Even at 10° and 40°, however, the difference amounts to only 0.15 gram of protein per 100 ml plasma; hence the errors caused by using the previous standard solutions at even such extreme temperatures were not important.

If only enough stock solution is required for a field set of standard solutions, 42.5 gm of sulfate is placed in a bottle or flask holding 300 ml or more. A 250 ml volumetric flask is filled with the volume of water indicated by the last column of table XIX. The water is emptied onto the crystals, the flask drained 2 minutes, and the crystals dissolved, as directed above.

tration and transfer the solution to the storage bottle as before. Rinse, refill, and empty the 1-liter flask twice more in the same manner, obtaining 4 liters of the stock solution.

(5) *Preparation of sets of standard solutions.*

(a) *Complete laboratory set in 100 ml portions.*

1. For the standard of 1.075 gravity, measure 74 ml of stock solution from a burette into the 100 ml volumetric flask, and fill the flask to the mark with water. Mix the solution, transfer it to a labeled 120-ml bottle, and stopper it to prevent evaporation.
2. To prepare the standard of gravity 1.074, rinse the 100 ml flask once with water, and refill the burette from a 250 ml Erlenmeyer flask containing the stock solution. Then measure 73 ml of the stock solution into the volumetric flask and dilute to 100 ml.
3. Carry a like procedure through for preparation of the entire series. For each standard the number of ml of stock solution *less by 1* than the number indicated in the second and third decimal places of the desired gravity is measured into the rinsed 100-ml flask and diluted to the mark.
4. If there were no contraction when the stock solution is mixed with water one would dilute 75 ml of the stock to 100 ml to get a gravity of 1.075, 74 ml to get 1.074, etc. Since there is a contraction, this is empirically corrected by taking 1 ml less of the stock solution. It happens conveniently that the same 1 ml correction serves for the entire range, 1.075 to 1.008, over which its use yields gravities correct within ± 0.0003 .
5. If, for purposes of special precision, gravities exact to 0.0001 are desired, the standards are made up according to table XXI. The convenient rule of thumb outlined above (1 ml less than the number indicated by the decimal figure of the desired gravity) suffices for ordinarily required accuracy.

(b) *Complete laboratory set in 50-ml or 25-ml portions.* Portions of 25 or 50 ml are prepared by dilution of the volumes indicated in table 3 to 25 or 50 ml in volumetric flasks of these capacities.

(c) *Field set for approximate blood and plasma analyses.* Sixteen standard solutions covering the range 1.016 to 1.076 in steps of 0.004 are prepared.

- (d) *Pocket field set for whole blood only.* Six standard solutions of gravities 1.040, 1.046, 1.052, 1.058, 1.064, and 1.070 are prepared in 30 ml bottles.

d. DRAWING OF BLOOD.

- (1) *Venous blood.* Tourniquets should not be applied for more than 1 minute. Longer application may force so much fluid out of the blood that the concentrations of both plasma proteins and hemoglobin are measurably increased.
- (2) *Capillary blood.*
 - (a) Capillary blood may ordinarily be used for hemoglobin estimations from the specific gravity of the whole blood.
 - (b) To draw capillary blood, a finger, preferably the fourth or ring finger, is cleansed with alcohol, wiped dry, and is pricked with a sterile needle or stylette. The puncture must be forceful, as a fairly large drop of blood is necessary. The size of the drop may be increased by massaging the finger from the base toward the tip. A "vaccine" capillary tube or a blood-counting pipette is applied to the blood drop while the other end of the tube is held at a level slightly lower. As the blood enters the tube, care is taken to keep the tip of the capillary well immersed in the drop in order to prevent the entrance of bubbles of air.
 - (c) Enough blood may be collected in this way to provide 3 or 4 drops for the copper sulfate test. A single drop suffices to tell whether the hemoglobin is above or below a given standard, as in tests of blood donors. The blood is immediately used for the test, before there is time for clotting.

e. HANDLING OF VENOUS BLOOD.

- (1) *Without anticoagulant.* If the specific gravity of whole blood only, or of whole blood and serum, is to be measured, no anticoagulants are necessary. The blood is drawn into a syringe, and dropped directly from the syringe needle into the copper sulfate solutions to determine whole blood gravity. Then the remainder of the blood is transferred to a centrifuge tube and allowed to clot in order to obtain serum for use in methods described below.
- (2) *With anticoagulant.*
 - (a) If quick results for both whole blood and plasma are desired, an anticoagulant is added in order to permit

immediate centrifugation. The routine procedure is to transfer 5 ml of the blood from the syringe to a test tube containing 5 mg of the ammonium-potassium oxalate mixture. Larger proportions of oxalate must not be used, as they increase the gravity of the blood and plasma. Heparin (0.2 mg per ml of blood) may be used in place of oxalate; it is the ideal anticoagulant since it has no significant effect on the gravity. Part of the blood may be used to measure whole blood gravity. The rest is at once centrifuged to obtain plasma. During centrifugation the tubes should be capped to prevent evaporation.

- (b) The gravity of the whole blood may be determined either immediately after drawing, by dropping blood from the syringe needle into copper sulfate standards, or after transfer to the oxalated tube, before centrifugation. In the latter case *it is necessary to mix the cells and plasma thoroughly* before the blood sample is taken from the oxalated tube into a dropper or syringe. To attain complete mixing either invert the tube containing the oxalated or heparinized blood 10 times, or stir with a glass rod with a mushroom end, which is raised and lowered through the blood 10 times, just before the sample is drawn into the dropper or syringe. Gross error in hemoglobin estimation could result if the blood sample were taken from blood in which partial settling of the cells had occurred.

f. DETERMINATION OF SPECIFIC GRAVITY.

- (1) The drop of serum, plasma, or whole blood is delivered from a height of about 1 cm²³ above the solution from a medicine dropper, a capillary, or a syringe needle. It is preferable to use small drops for the reason that they permit more tests before the standard solution must be changed. Therefore a medicine dropper with a fine tip is preferable to one with a coarse tip. Greasing the outside of the tip with vaseline also reduces the size of the drop, especially if the vaseline is mixed with a little caprylic alcohol. When the drop is delivered it is convenient to steady the dropper on the edge of the bottle. (See fig. 20.)

²³ If the drop falls from too great a height it may be broken up on striking the solution, or its momentum may carry it too far below the surface. On the other hand, if it strikes with too little force the drop may not break through the surface film. A fall of about 1 cm gives the right striking force.

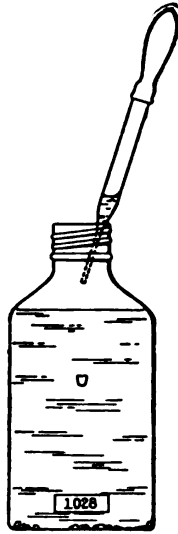


Figure 20. Method of delivering a drop of serum, plasma, or whole blood into a bottle of standard copper sulfate solution.

- (2) The delivered drop breaks through the surface film of the solution and penetrates 2 or 3 cm below the surface; within 5 seconds the momentum of the fall is lost, and the drop then either begins to rise, becomes stationary, or continues to fall. The gravity of the drop relative to the solution does not change appreciably until the drop has been immersed in the solution for another 10 or 15 seconds, and there is ample time to note its behavior during this interval. If the drop is lighter than the test solution it will rise, perhaps only a few millimeters, and may begin to sink immediately afterward. If the drop is of the same gravity as the standard test solution it will become stationary for this interval and then fall. If the drop is heavier it will continue to fall during the interval. *In summary, the behavior during the 10 seconds after the drop has lost the momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution; if it rises at all during this period it is lighter than the standard.*

Example: The following example shows how, by bracketing on the probable extremes of a plasma's gravity range and then testing intermediate points, one can determine the correct specific gravity to within ± 0.0002 , with not more than 4 drops.

The plasma was expected to be of normal or greater concentration. Four successive drops gave the following results, in which the figures indicate the gravities of the

standards, and + or — indicate that the plasma was heavier or lighter than the preceding standard: 1.027,+; 1.031,—; 1.029,+; 1.030,—. The plasma was heavier than 1.029 and lighter than 1.030, and could therefore be placed at 1.0295, with an error not greater than ± 0.0004 .

- (3) By noting the relative rate of fall or rise in the two adjacent solutions, 1.029, and 1.030, it was further obvious that the plasma was nearer 1.029 than 1.030. Being less than 1.0295 and greater than 1.0290 it could be placed at 1.0293, with an error not greater than -0.0002 .

g. APPROXIMATE FIELD DETERMINATIONS. For field work it may suffice to determine the gravities to ± 0.001 . For this only 16 standard solutions with gravity intervals of 0.004 covering the range from 1.016 to 1.076 are needed. An error of 0.001 in plasma gravity affects plasma proteins by 0.3 gm per 100 ml, additive errors of 0.001 in the gravities of both plasma and whole blood affect hemoglobin results by a maximum of 5 percent.

Example: The plasma tested was lighter than 1.028 and heavier than 1.024. By observing the behavior of the drop in the two solutions it was noted to be closer to 1.028 than to 1.024 and hence could be placed at 1.027, with an error not greater than ± 0.001 .

With the pocket set of 6 standard solutions (1.040, 1.046, 1.052, 1.058, 1.064, and 1.070) one can measure whole blood gravities to ± 0.002 .

h. CALCULATIONS OF PLASMA PROTEIN, HEMOGLOBIN, AND HEMATOCRIT FROM SPECIFIC GRAVITIES OF BLOOD AND OF PLASMA OR SERUM.

(1) *Line charts.*

- (a) Line charts for these calculations are given in figures 21 and 22. The calculations are made by laying a straight edge, preferably a transparent ruler, or a stretched thread, as directed on the charts.
- (b) For men, the normal ranges indicated on the line charts are taken from precise measurements made on the blood and plasma of 20 normal men. The normal hemoglobin range, in gm per 100 ml, is higher than that usually given in the literature, because the methods heretofore in most general use have given somewhat low results. For women the normal hemoglobin and cell volume are taken as 90 percent of the normal for men.
- (2) *Correction for use of too much oxalate anticoagulant.* The Heller and Paul oxalate mixture in the amount prescribed, one mg per ml of blood, increases the gravities of both plasma and whole blood by 0.0004. The effect is

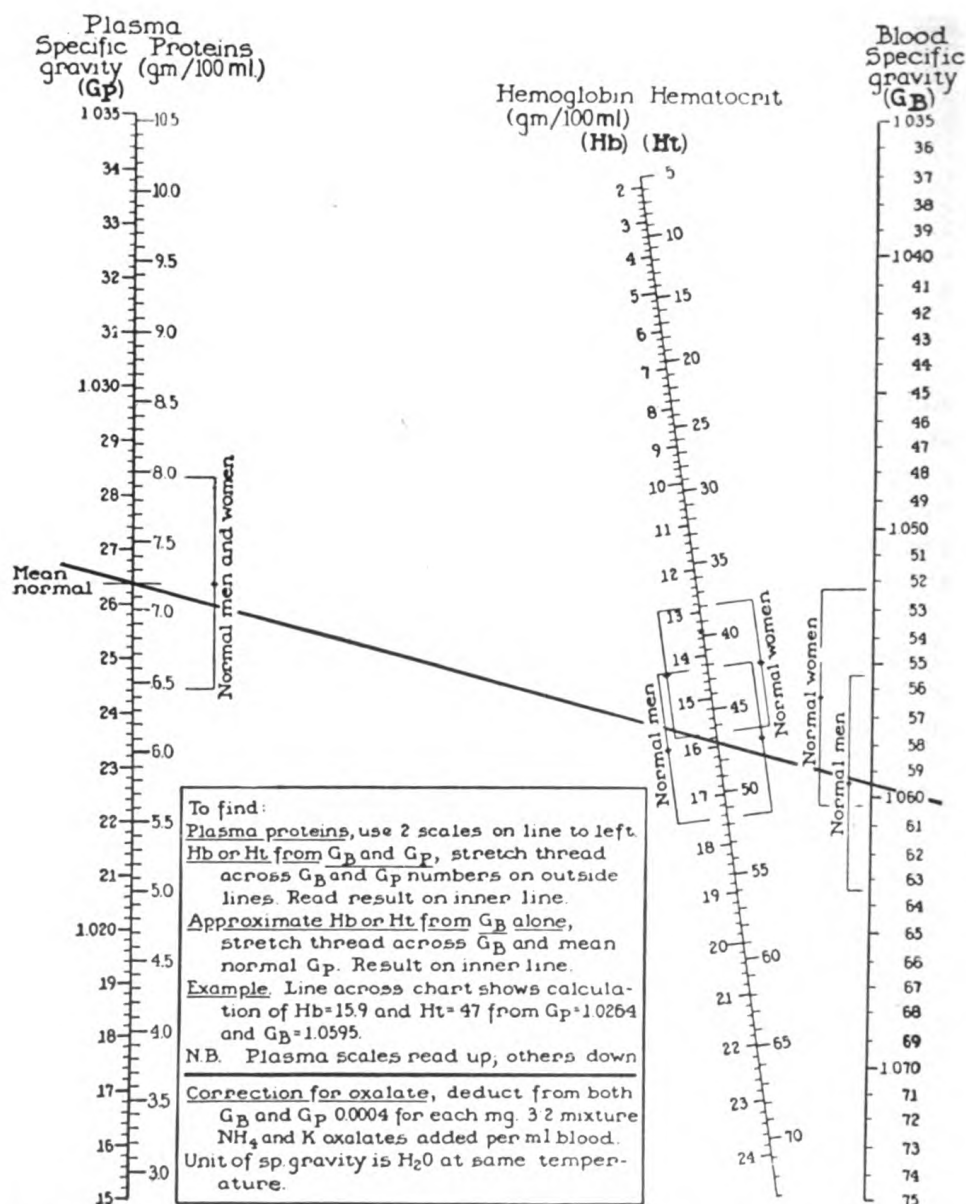


Figure 21. Line chart for calculating plasma proteins, hemoglobin and hematocrit from gravities of plasma and blood.

negligible for most purposes; it raises the calculated plasma protein and hemoglobin values each about 0.1 gm per 100 ml. When, however, less than 5 ml of blood are added to a tube prepared as directed for 5 ml of blood, with 5 mg of oxalate mixture, corrections should be made by subtracting from the observed gravities of both plasma and whole blood 0.0005 if the blood volume is 4

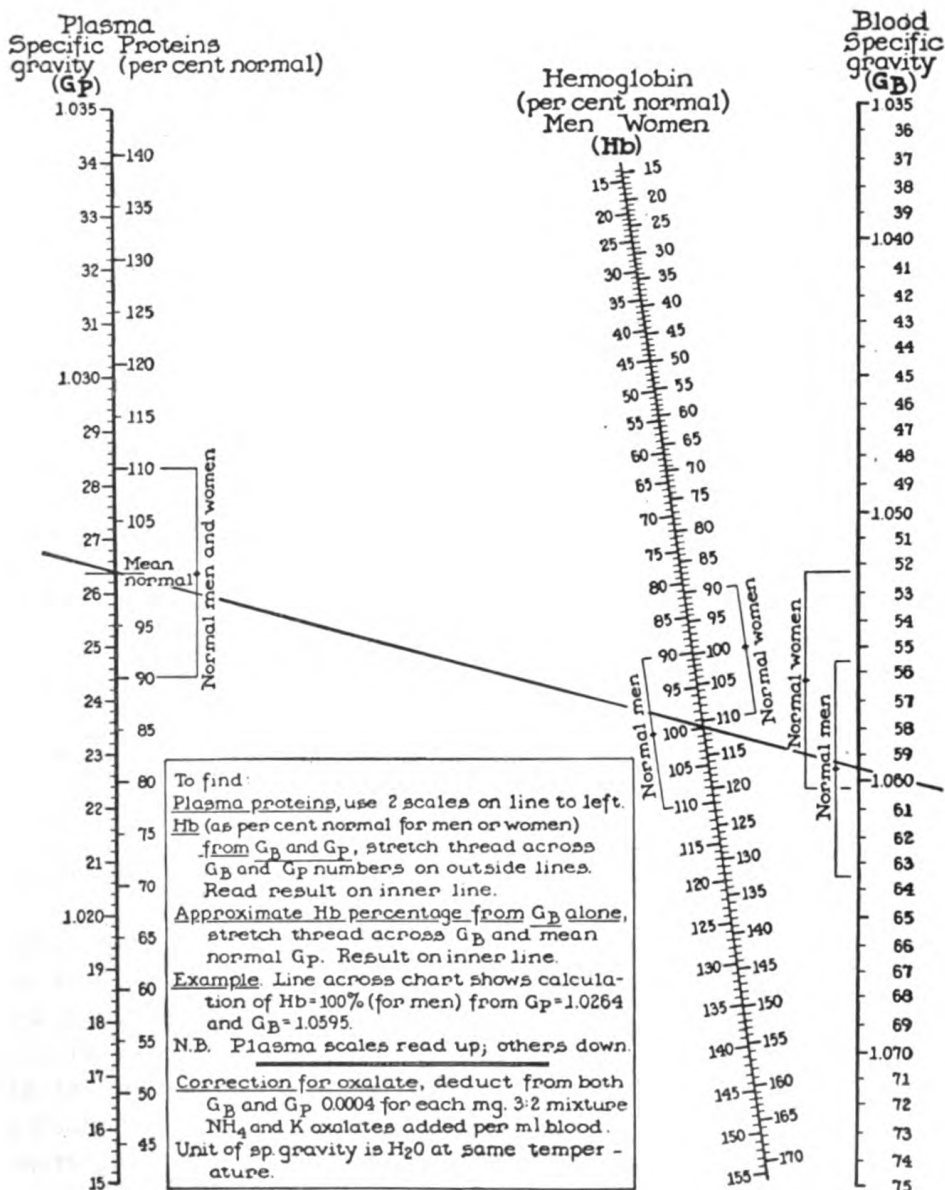


Figure 22. Line chart for calculating percentages of normal plasma proteins and hemoglobin from gravities of plasma and blood.

ml, 0.0007 if it is 3 ml, 0.0010 if it is 2 ml, and 0.0020 if it is 1 ml.

- (3) *Correction for use of serum instead of plasma.* The fibrinogen of plasma is ordinarily 0.2 to 0.3 gm per 100 ml. Removal of the fibrinogen makes the gravity of the serum about 0.0005 lower than that of plasma. Hence, when gravity is determined on serum add 0.0005 to the observed specific gravity to estimate the gravity of the plasma.

- (4) *Equations.* The equations given below can be used to calculate results if the line charts, which are based on these equations, are not available. G_P indicates specific gravity of plasma (water being taken as unity at the same temperature); G_B indicates gravity of whole blood; 1.0970 is the mean specific gravity of the cells; 46.1 is the mean O_2 capacity of cells, 33.9 the mean gm of hemoglobin per 100 ml of cells.

$$\text{Plasma proteins (gm per 100 ml blood)} = 370 (G_P - 1.0070). \quad (1)$$

$$\text{Hematocrit (ml cells in 100 ml blood)} = 100 \times \frac{G_B - G_P}{1.0970 - G_P}. \quad (2)$$

$$\text{Oxygen capacity (ml } O_2 \text{ bound by 100 ml blood)} = 46.1 \frac{G_B - G_P}{1.0970 - G_P}. \quad (3)$$

$$\text{Hemoglobin (grams per 100 ml blood)} = 33.9 \frac{G_B - G_P}{1.0970 - G_P}. \quad (4)$$

In equation 1 the constant, 370, replaces the value 343, used in the literature and in previous publications of this method; 370 has been found more exact, as the result of comparison of plasma gravities with proteins determined by accurate macrokjeldahl analyses.

i. SPECIAL POINTS AND PRECAUTIONS.

- (1) *Utility of whole blood gravity alone without plasma or serum gravity.*

(a) For accurate estimation of hemoglobin or cell concentration it is necessary to measure the gravities both of whole blood and of plasma because, as indicated by equation 4, the accurate calculation of the hemoglobin is based partly on the difference, $G_B - G_P$, the extent by which the whole blood gravity exceeds the plasma gravity. However, when information concerning gross changes of blood concentration is desired, and facilities or time is lacking to obtain plasma or serum, useful information can be obtained from the gravity of the whole blood alone. From it one can note in which direction concentration change is moving. And one can make an approximate estimate of the hemoglobin concentration with the line charts by using as plasma gravity the average normal, 1.0264.

(b) When Hb is estimated from G_B alone, the error, caused by deviations of G_P from the assumed 1.0264, varies inversely as the gravity of the whole blood. If G_B is above 1.050, the error, caused by the usual maximal deviation of ± 0.0018 of G_P from its normal mean, is

less than 0.6 gm of hemoglobin per 100 ml of blood; and the error caused by variations of G_p over the range 1.031 and 1.021 (which include most, but not all, pathological plasmas), does not exceed ± 1.6 gm of Hb per 100 ml, or ± 10 percent of the average normal Hb for men.

- (c) If, however, the whole blood gravity is below 1.050, the effects of plasma variation on the calculated hemoglobin are greater. Consequently if a G_B value is found below 1.050, it is essential to determine also the G_p for calculation of Hb.
 - (d) In a few conditions, including myeloma, kala-azar, and lympho-granuloma venereum, the globulins are so increased that the plasma gravity exceeds 1.031. Such hyperglobulinemia appears to occur in about 1 percent of the usual run of hospital patients. In such cases, hemoglobin calculated from G_B alone erroneously includes, as hemoglobin, the excess plasma protein above 7 gm per 100 ml. The plasma proteins may, in rare cases, be as high as 12 percent. A gross plus error could occur in such cases in the Hb if estimated from G_B alone.
 - (e) If such cases are excluded, and those with blood gravities below 1.050, whole blood gravities can serve for rapid approximate routine estimations of hemoglobin from drops of finger blood.
 - (f) When routine hemoglobin estimations are made from whole blood gravities, it is convenient to prepare a set of standard copper sulfate solutions indicating hemoglobin percentages at intervals of 10, and label the bottles or tubes of standard solutions with the hemoglobin percentages, so that no calculations are required. The gravity determinations are made as described above for field determinations. The standard solutions may be made as indicated in table XXII.
 - (g) In blood bank stations of the American Red Cross (1944) a single standard copper sulfate solution was used, of gravity 1.052, indicating 12.2 gm of hemoglobin per 100 ml of blood. Only subjects with whole blood gravity above 1.052 were accepted as donors. The test was made with 1 drop of finger blood (Thalhimer).
- (2) *Number of analyses that can be made before renewal of copper sulfate standard solutions is necessary.*
- (a) Tests have shown that a standard solution will receive about one-fortieth its volume of plasma or blood, or 1

Table XXI. Ml of Stock Copper Sulfate Solution of Gravity 1.100 to be Diluted to 100 ml, 50 ml, or 25 ml to Prepare Standard Solutions of Gravity, G, to Within 0.0001

G	100	50	25	G	100	50	25
1.008	7.33	3.67	1.84	1.038	37.0	18.50	9.25
9	8.32	4.16	2.08	39	38.0	19.00	9.50
10	9.31	4.66	2.33	40	39.0	19.50	9.75
11	10.30	5.15	2.58	41	40.0	20.00	10.00
12	11.29	5.65	2.83	42	41.0	20.50	10.25
13	12.28	6.14	3.07	43	42.0	21.00	10.50
14	13.27	6.64	3.32	44	43.0	21.50	10.75
15	14.26	7.13	3.57	45	44.0	22.00	11.00
16	15.25	7.63	3.82	46	45.0	22.50	11.25
17	16.24	8.12	4.06	47	46.0	23.00	11.50
18	17.23	8.62	4.31	48	47.0	23.50	11.75
19	18.22	9.11	4.56	49	48.0	24.00	12.00
20	19.21	9.61	4.81	50	49.0	24.50	12.25
21	20.20	10.10	5.05	51	50.0	25.00	12.50
22	21.19	10.60	5.30	52	51.0	25.50	12.75
23	22.17	11.09	5.56	53	52.0	26.00	13.00
24	23.15	11.58	5.79	54	53.0	26.50	13.25
25	24.94	12.07	6.04	55	54.0	27.00	13.50
26	25.12	12.55	6.28	56	55.0	27.50	13.75
27	26.10	13.05	6.53	57	56.0	28.00	14.00
28	27.08	13.54	6.77	58	57.0	28.50	14.25
29	28.06	14.03	7.02	59	58.0	29.00	14.50
30	29.04	14.52	7.26	60	59.0	29.50	14.75
31	30.0	15.01	7.51	61	60.0	30.00	15.00
32	31.0	15.50	7.75	62	61.0	30.50	15.25
33	32.0	16.00	8.00	63	62.0	31.00	15.50
34	33.0	16.50	8.25	64	63.0	31.50	15.75
35	34.0	17.00	8.50	65	64.0	32.00	16.00
36	35.0	17.50	8.75	66	65.0	32.50	16.25
37	36.0	18.00	9.00	67	66.0	33.00	16.50
				68	67.0	33.52	16.76
				69	68.1	34.04	17.02
				70	69.1	34.56	17.28
				71	70.2	35.08	17.54
				72	71.2	35.60	17.80
				73	72.2	36.12	18.06
				74	73.3	36.64	18.32
				75	74.3	37.15	18.58

In the columns under 100, 50, and 25 are given the ml of stock solution that must be diluted to 100, 50, or 25 ml to form standards of the indicated G values.

Table XXII. Standard Copper Sulfate Solutions for Estimating Hemoglobin Percentages from Observations on Whole Blood, with Assumed Plasma Gravity of 1.024

Percentage of normal hemoglobin		G_H Gravity of whole blood	Ml of stock copper sulfate solution per 100 ml of standard solution
Men	Women		
30	33	1.0365	35.5
40	44	1.0400	39.0
50	55	1.0431	42.1
60	67	1.0465	45.5
70	78	1.0495	48.5
80	89	1.0530	52.0
90	100	1.0563	55.3
100	111	1.0595	58.5
110	122	1.0629	61.9
120	133	1.0662	65.2

small drop per ml, under the conditions of the tests, before the gravity of the standard is changed by 0.0005. The change is to decrease for gravity. A 100 ml portion of standard serves for about 100 tests.

- (b) When whole blood is tested in the copper sulfate solution some hemolysis occurs, the color of the solution shifts from blue toward green, and it becomes slightly turbid from suspended unlaked cells. However, the gravity of the solution is not changed by more than 0.0005 until one-fortieth its volume of blood has been added.
- (c) While a standard solution of 100 ml volume must be replaced after receiving about 100 drops of blood or plasma, analysis of 100 bloods leaves most of the solutions of a set but little used. As a rule, only the solutions with gravities within the normal ranges of blood and plasma will require replacement. The rest of the standard solutions, since they are used only when abnormal blood is encountered, will usually not require replacement before 200 or more bloods and their plasmas have been analyzed.
- (d) In order to tell when the solutions in the bottles should be changed, prepare for comparison two extra standard solutions of gravity about 1.028 and 1.060, in bottles of the same size used for the standard set. To the solution of gravity 1.028 add one-fortieth its volume of normal plasma, and to the solution of gravity 1.060 add one-fortieth its volume of normal whole blood. These control bottles are kept for comparison with used

standards. When the volume of precipitate in the bottom of a standard equals that in the control bottle the standard is renewed.

- (e) If, for any reason, it is impossible to renew the standard solutions after they have been used often enough to decrease their gravity by 0.0005, each such standard may be relabeled with a gravity 0.001 lower and used further. Thus, after 50 small drops have been added to 50 ml of a 1.028 standard in a 2-ounce bottle, the standard can be relabeled 1.027. This is not a desirable procedure, but it may be resorted to in an emergency.
 - (f) The use of 5 ml portions of standard solutions in vials or Kahn tubes has been described in paragraph 215b.
- (3) *Surface film effects in the analyses.*
- (a) Occasionally a drop will fail to make a clean break through the surface film of the copper sulfate solution, and remain attached by a tentacle to the film. In this case the drop is detached from the film by tapping the tube, and a fresh drop is tried.
 - (b) After each test, one makes sure that the surface film is left clean and free from fragments. If any are left on the film they are likely to prevent a clean breakthrough of the drop in the next tests. Fragments caught in the surface film can usually be detached by tapping the tube; they then sink to the bottom. Sometimes, however, a fragment of fatty nature or holding a bubble will continue to float on the surface. Such fragments are removed with a wooden applicator stick.
- (4) *Temperature effects.* Because the copper sulfate standard solutions have almost exactly the same coefficients of expansion as blood and plasma, no corrections for temperature are needed. Freshly drawn blood can be dropped from the syringe directly into the copper sulfate standard solutions at room temperature without significant error, because the drop of blood quickly takes the temperature of the surrounding copper sulfate solution. Convection currents in the standard solutions could introduce false readings. Do not bring cold bottles into a warm room and use at once. Do not leave bottles near stove, on window sill, etc. Hold bottles only by top when using, not by sides.
- (5) *Accuracy of method.*
- (a) *Plasma proteins.* The proteins per 100 ml of plasma calculated from specific gravities are within ± 0.4 gm of proteins calculated from accurate macrokjeldahl

analyses (adequate digestion with mercury as catalyst) in normal and most pathological plasmas. When blood urea is very high (urea N over 100 mg per 100 ml) the gravity-calculated proteins are likely to be somewhat too high, because the non-protein constituents contribute more to the gravity than is allowed for in the term 1.007 of equation 1. In extreme cases of this type the gravity-calculated protein may be as much as 1 gm per 100 ml too high (blood urea N over 200 mg per 100 ml) but when the blood urea N is under 150 mg the error in gravity-calculated protein is usually under 0.6 gm. Shock and allied conditions in man does not seem to effect seriously the accuracy.

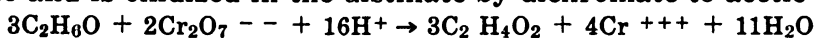
- (b) *Hemoglobin.* In normal blood, and in pathological bloods in which the hemoglobin concentration within the red cells is not affected, the hemoglobin content, calculated by the line-charts from G_B and G_P has been found regularly within 2 percent of the value measured by oxygen capacity, or within ± 0.3 gm of hemoglobin per 100 ml. In pathological blood conditions, such as pernicious anemia, the error can be larger, but has not been found greater than ± 0.6 gm of hemoglobin per 100 ml. The calculation of hemoglobin from gravities by equation 4 assumes a constant cell gravity of 1.0970 and a cell hemoglobin content of 33.9 gm per 100 ml. That the gross deviations from these values that can occur in disease do not more affect the accuracy of the hemoglobin determination, is due to the fact that changes in the hemoglobin content of the cells change the cell specific gravity in the same direction, and the effects on the two assumed constants nearly cancel each other in calculating the hemoglobin content of the blood.
- (c) *Hematocrit.* In the hematocrit calculation (p. 302, equation 2) there is no cancellation of the effects of variations in the hemoglobin concentrations in the cells as there is in equation 4. Hence the percentage error in calculation of hematocrits from gravities is greater than in calculation of hemoglobin. However, hematocrit figures are accurate enough to be clinically useful, except in diseases where the abnormality of hemoglobin concentration in the cells is great. In bloods with normal concentrations of hemoglobin in their cells the gravity method indicates the hematocrit value within 2 ml per 100 ml of blood. In blood with

markedly abnormal cells the error may be 2 or 3 times as great. Shock and allied conditions do not appear to increase the error above that found in normal blood.

216. Ethyl Alcohol in Blood and Cerebrospinal Fluid

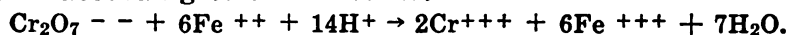
(Harger, J. Lab. Clin. Med., 20, 746, (1934-35))

a. GENERAL. The alcohol (ethanol) is distilled from blood filtrate and is oxidized in the distillate by dichromate to acetic acid:



The oxidation is carried out with a measured amount of standard dichromate solution, and the excess dichromate left after the oxidation is titrated, the decrease in dichromate caused by the alcohol being the measure of the latter. One mole of ethanol is oxidized by $2/3$ mole, or $2/3 \times 6 = 4$ gram-equivalents (par. 138), of $\text{Cr}_2\text{O}_7^{--}$. Hence 1 mg of alcohol ($1/46.1$ millimole) is oxidized by 1 ml of dichromate solution of $4/46.1$, or 0.0868, normality. In the method the dichromate solution used is 0.0434 *N*, of which one ml is equivalent to 0.5 mg of ethanol.

For titration of the dichromate a standard solution of ferrous sulfate and methyl orange is used. The Fe^{++} is oxidized in the titration according to the reaction:



The methyl orange is also oxidized instantly by the dichromate, so that the red dye is replaced by a relatively colorless oxidation product. When the ferrous sulfate-methyl orange solution is added to dichromate solution the red color of the added methyl orange disappears as soon as the dye is mixed with the dichromate solution until all the dichromate has been reduced by the combined amounts of Fe^{++} and methyl orange added. Then the addition of another drop of the Fe^{++} -methyl orange solution gives the titration mixture a pink color, due to undestroyed methyl-orange.

b. APPARATUS.

- (1) *Erlenmeyer Flasks, 50 ml.*
- (2) *Microburette.* One of 5 ml capacity.
- (3) *Distilling flask, 250 ml.*
- (4) *Condenser.* A condenser with a straight glass condensing tube.
- (5) *Volumetric mixing cylinder, graduated and glass-stoppered, 50 ml.*
- (6) *Beads or fragments of glass or Vitreosil to prevent bumping.*

c. REAGENTS.

- (1) *Concentrated sulfuric acid, A.C.S.*
- (2) *Approximately 17 N sulfuric acid.* Pour, slowly with

stirring, 1 volume of the concentrated sulfuric acid into one volume of water and cool.

- (3) *Methyl-orange, 0.1-percent solution.* Dissolve one gm of methyl-orange in 1 liter of approximately 0.025 N NaOH solution.
- (4) *Ferrous sulfate, 20-percent solution.* Place 50 gm of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, weighed to 0.1 gm, in a 250-ml volumetric flask. Dissolve the sulfate in 150 ml of water. Add 30 ml of concentrated sulfuric acid, with stirring, and dilute to 250 ml.
- (5) *Ferrous sulfate-methyl-orange standard solution.* Place in a 125-ml Erlenmeyer flask 70 ml of the 17 N sulfuric acid. Add 30 ml of the methyl-orange solution and 2 ml of the 20-percent ferrous sulfate solution. The solution is approximately 0.0144 N with respect to Fe, but the additional reducing effect of the methyl orange makes the total reducing power approximately 0.0174 N, so that about 2.5 ml titrate 1 ml of the 0.0434 dichromate. The solution deteriorates on standing; it can be used for 3 or 4 days after preparation, but must be standardized against dichromate, by performing the blank titrations described below, on the same day that it is used for analysis.
- (6) *Standard dichromate solution, 0.0434 N.* Weigh 2.129 gm of potassium dichromate to 0.1 mg, transfer to a 1-liter volumetric flask, dissolve in water, and make up to a liter.
- (7) *The 10-percent sodium tungstate solution and N/12 sulfuric acid* for blood protein precipitation in paragraph 190.

d. PROCEDURE.

- (1) Precipitate the proteins in 2 or more ml of blood as described in paragraph 190, to obtain a 1:10 filtrate. Pipette 10 ml of the filtrate into the 250-ml distilling flask. Add 40 ml of water and some glass beads. Connect the distilling flask with the condenser, and arrange the outlet of the latter so that it will dip nearly into the middle of a 50 ml stoppered graduated cylinder. Heat the distilling flask with a small flame and distill until 25 ml of distillate has collected in the cylinder. Then add water to the cylinder until it is filled to the 50-ml mark, stopper, and mix the contents by repeated inversion.
- (2) For oxidation of the alcohol, measure with a transfer pipette 10 ml of the diluted distillate into 50-ml Erlenmeyer flask, add 2 ml of the 0.0434 N dichromate solu-

tion, then 10 ml of concentrated sulfuric acid. Mix well by rotating the flask and let stand for 10 minutes. The heat developed by the mixing of the acid and water accelerates the oxidation of the alcohol. At the end of exactly 10 minutes cool in water to room temperature. Titrate the remaining dichromate with the ferrous sulfate-methyl orange solution. During the titration the yellow of dichromate fades as the $\text{Cr}_2\text{O}_7^{--}$ is reduced. Then, when the first drop of excess reducing solution is added, the pink color of the methyl orange fills the solution and makes a sharp end-point.

- (3) Perform one blank titration, B_1 , on 10 ml of water, plus 1 ml of 0.0434 N dichromate, plus 10 ml of sulfuric acid, and a second blank, B_2 , like B_1 except that 2 ml of the dichromate solution are used in B_2 .

e. CALCULATION:

$$(1) \text{ Mg ethanol in sample titrated} = 0.5 \times \frac{B_2 - U}{B_2 - B_1}.$$

$$(2) \text{ Mg ethanol per 100 ml of blood} = 250 \times \frac{B_2 - U}{B_2 - B_1}.$$

B_1 is the ml of ferrous sulfate-methyl orange used to titrate the blank containing 1 ml of dichromate, B_2 the ml to titrate the blank containing 2 ml of dichromate, and U the ml to titrate the unknown after reaction with alcohol.

$B_2 - U$ indicates the amount of dichromate reduced by the alcohol, when the dichromate is measured in terms of its equivalent in ml of the ferrous sulfate-methyl orange solution. $B_2 - B_1$ indicates the ml of this solution that are equivalent to 1 ml of the 0.0434 N dichromate. Hence $(B_2 - U)/(B_2 - B_1)$ indicates the ml of 0.0434 N dichromate reduced by the alcohol. $B_2 - B_1$, rather than B_1 is taken to indicate the ml of ferrous sulfate-methyl orange that are equivalent to 1 ml of dichromate for the reason that the distilled water and the sulfuric acid may contain traces of reducing material, which would make B_1 lower than the true equivalent volume. The use of the value $B_2 - B_1$ to standardize the ferrous sulfate-methyl orange solution automatically prevents error from such material.

If there is so much alcohol that it reduces almost the entire 2 ml of dichromate, so that U is less than 0.1 ml, repeat the titration to determine U , but use a portion of distillate less than 10 ml, and add enough water to make 10 ml before adding the sulfuric acid. The alcohol is thus calculated as:

$$(3) \text{ Mg ethanol per 100 ml blood} = \frac{2500}{V} \times \frac{B_t - U}{B_t - B_i}.$$

V is the ml of distillate used for the analysis.

f. It is to be remembered that this test will also reduce dichromate if methanol is present. Therefore, every specimen, which shows reduction, should be tested for methanol qualitatively. This may be done as follows:

- (1) Make a tight coil of copper wire (16 gauge) $1\frac{1}{2}$ inches in length by wrapping it around a pencil. Leave a straight 8" strip at one end to be used as a handle after inserting it into a cork. Place 5 ml of distillate in a test tube. Heat the copper spiral till it glows and plunge into the distillate. Repeat this six times. Carefully add 1 ml of concentrated sulfuric acid and cool. Add 0.5 ml Schiff's reagent (see (2) below). A pink or violet color appearing within 10 minutes indicates formaldehyde. Acetaldehyde does not give this reaction in the presence of concentrated sulfuric acid.
- (2) Schiff's reagent: To 500 ml water add 0.5 gm of basic fuchsin. Bubble sulfur dioxide through this until the weight has increased by 5 gm. Let stand overnight to dissolve as much of the fuchsin as possible. Dilute up to one liter. Add 1 gm of decolorizing carbon or activated charcoal and mix well. Filter. This procedure should remove any discoloration in the solution. Repeat if necessary. The final solution must be white. Store in a glass-stoppered bottle where it will keep indefinitely.
- (3) Should the test for methanol be positive it will not be possible to determine quantitatively the relative amounts of methanol and ethanol without resorting to special methods.

g. *Ethyl alcohol in cerebrospinal fluid.* Prepare a 1:5 protein free filtrate according to the directions given in paragraph 190b(2). To 2.5 ml of filtrate add an equal amount of distilled water and proceed in the same manner as outlined for blood filtrate.

h. An alternative simpler method for determining ethanol is a modification of Nicloux's procedure. This may be found in Simmons and Gentzkow, *Laboratory Methods of the United States Army*, Fifth Edition, Lea and Febiger.

217. Sulfonamides in Blood by Method of Bratton and Marshall (J. Biol. Chem., 128, 537, (1939))

a. GENERAL.

- (1) The method is based on diazotization of the NH_2 group of the drug and subsequent coupling with N (1-naphthyl)-ethylenediamine to form an azo dye, which is measured in a colorimeter or photometer. The procedure is identical for sulfanilamide, sulfathiazole, sulfadiazine, or sulfaguanidine, although the different sulfonamides yield colors which vary somewhat in intensity.
- (2) When the special reagents for this method are available it has over the alternative method of Fuller described in paragraph 218 the advantage that the purple color of the Bratton-Marshall method is somewhat better for visual reading, and permits sulfonamides to be measured, either visually or by electrophotometer, in about half the concentration required by the yellow color obtained in the Fuller method. Hence the Bratton-Marshall method can be carried out with smaller blood samples.
- (3) It is usually important to determine both the free and the total (free + conjugated) sulfonamide. Some patients acetylate a large part of the sulfonamide in their blood. In such a case if only the free drug were measured it would appear that absorption was incomplete, while if only the total drug were measured the lack of free drug in the circulation would be overlooked. The free drug has the therapeutic effect, while the acetylated drug may be the form more likely to cause renal damage by crystallizing in the kidneys.

b. REAGENTS.

- (1) *Trichloroacetic acid, 15-percent solution.* Dissolve 150 grams in water and dilute to 1 liter.
- (2) *Stock sodium nitrite solution, 50 percent.* Dissolve 50 gm of sodium nitrite in 80 ml of distilled water. The mixture makes 100 ml of solution. Store in a brown glass bottle or in a dark closet. The solution is stable.
- (3) *Sodium nitrite, 0.1-percent solution.* One ml of the 50-percent solution is diluted to 500 ml with distilled water. Prepare the solution fresh on the day it is to be used.
- (4) *N-(1-naphthyl)-ethylene-diamine dihydrochloride, 0.1-percent solution.* Dissolve 100 mg of the dihydrochloride in water and dilute to 100 ml. Keep the solution in a dark bottle. If kept in an ice box when not in use it will keep

for a week. If it must be kept at room temperature, prepare the solution on the day it is to be used.

- (5) *Saponin, 0.05-percent aqueous solution.* This is needed if blood sulfanilamides are determined with a colorimeter, but is not needed if a photometer is used.
- (6) *Hydrochloric acid, 4 N solution.*
- (7) *Ammonium sulfamate ($\text{NH}_4\text{SO}_3\cdot\text{NH}_2$), 0.5-percent aqueous solution.*
- (8) *A stock standard solution containing 100 mg per liter of sulfanilamide, sulfapyridine, sulfadiazine, or sulfaguani-dine (whichever is to be determined). The chemically pure, dry, finely pulverized drug should be used, not tablets. The solutions can be kept for several months in an ice box.*
- (9) *Working standard solutions.* These contain 1, 0.5, and 0.2 mg of the drug per 100 ml. Measure portions of 10, 5, and 2 ml of the stock solution of the drug to be determined into 100-ml volumetric flasks. Add 18 ml of the 15 percent trichloroacetic acid to each flask and dilute with distilled water to 100 ml. The working standards should be freshly prepared on the day they are used.

c. PROCEDURE.

- (1) *Removal of proteins.*
 - (a) *For colorimetric analysis.* Two ml of oxalated blood is measured into a 100 ml Erlenmeyer flask and 30 ml of the saponin solution is added. The fluids are mixed and allowed to stand 2 minutes for the cells to dissolve. Then the proteins are precipitated by adding 8 ml of the trichloroacetic acid solution and mixing thoroughly. The mixture is centrifuged, or is filtered through a dry filter. Twenty ml of the filtrate represents 1 ml of blood.
 - (b) *For photometric analysis.* One ml of blood is measured into a 50-ml volumetric flask and is mixed with 35 ml of water. The mixture is allowed to stand a few minutes to dissolve the cells (saponin is unnecessary in this case because of the great dilution). Then 10 ml of the trichloroacetic acid solution is added and the volume is made up to 50 ml by addition of distilled water. The mixture is centrifuged or filtered through a dry filter.
- (2) *Development of color to measure free sulfonamide.* To 10 ml of either the 1:20 filtrate (colorimetric) or the 1:50 filtrate (photometric) add 1 ml of the 0.1-percent sodium nitrite solution, and mix. Let stand 3 minutes to

complete diazotization; then add 1 ml of the ammonium sulfamate solution to destroy the excess nitrous acid. Two minutes after adding the sulfamate add 1 ml of the naphthyl-ethylene-diamine solution. The color of the azo dye develops to a maximum in 3 minutes at 20°, and remains unchanged for 1 hour if the solution is kept in the dark.

- (3) *Development of color to measure total sulfonamide.* Ten ml of the 1:20 or the 1:50 blood filtrate is pipetted into a 15 by 150 mm test tube marked to contain 10 ml, and 0.5 ml of the 4 N hydrochloric acid solution is added. The open tube is then heated in a boiling water bath for 1 hour to set the conjugated sulfonamide free. The solution is cooled, and the volume, somewhat diminished by evaporation, is brought to 10 ml by addition of distilled water. The solution is then treated with nitrite, etc., as in determining the free sulfonamide.
- (4) *Development of color in standards.* For colorimetric analyses a 10-ml portion of each of the working standards is treated in the way described for treatment of blood filtrate to measure free sulfonamide. For photometric analyses only one standard, with 0.2 mg of sulfonamide per 100 ml, need usually be prepared.
- (5) *Colorimetric measurement.* Each filtrate is compared with the standard that it most nearly matches. The use of a green filter (Corning, Sextant 63, No. 401, 2 mm thick) facilitates the comparison, especially when the color is weak. It is convenient to set the 1 mg per 100 ml standard at 10 mm, the 0.5 mg standard at 15 mm, and the 0.2 mg at 20 mm. The color comparison can be made immediately after the color is developed, or can be deferred for as long as 1 hour if the solutions are kept in the dark.
- (6) *Colorimetric calculation.*

$$\text{Mg sulfonamide per 100 ml blood} = 20.6 \times C_s \times \frac{S}{U}$$

C_s is the mg of sulfonamide per 100 ml in the working standard, S is the reading of the standard, U the reading of the unknown. The factor 20.6 is used instead of the dilution factor 20, because precipitation of the blood proteins in the 1:20 dilution, used to prepare the filtrate for colorimetric analysis, involved the loss of about 3 per cent of the drug; this fraction is apparently adsorbed by

the precipitate of the blood proteins. Use of the factor 20.6 corrects for this loss.

(7) *Photometric measurement.*

(a) The blank is prepared from the reagents, with water in place of blood. The optical density of the blank increases with time if the solution is exposed to ordinary daylight. Some color-forming reaction appears to occur in light between the trichloroacetic acid and the N-(1-naphthyl)-ethylene-diamine. (Solutions of the diamine acidified with HCl instead of trichloroacetic acid do not develop color in light.) After addition of the naphthyl-ethylene-diamine reagent to either blanks or blood filtrates the photometric reading, should be made at once, or else the solution should be protected from light and the reading made within 1 hour.

(b) The optical density is measured with light of 540 millimicrons wave length.

(8) *Photometric calculation.*

$$\text{Mg sulfonamide per 100 ml blood} = 10 \times \frac{D_u}{D_s}$$

D_u is the optical density of the unknown, D_s the optical density of the standard with 0.2 mg of sulfonamide per 100 ml.

One may determine $k = \frac{10}{D_s}$ and use the k in subsequent analyses.

218. Sulfonamides in Blood by Method of Fuller

(Lancet, 115 (I), 194, (1937)); modified for the colorimeter by Jang and Cheng, Chinese Med. J., 61, 227, (1943) and for the photometer by Archibald (unpublished).

a. GENERAL. This method can be used when the special reagents, naphthyl-ethylene-diamine and ammonium sulfamate, required for Bratton and Marshall's method, are not available. Also, trichloroacetic acid, for precipitating blood proteins, can if necessary be replaced by alcohol in Fuller's method. The principle of forming an azo-dye is the same as in Bratton and Marshall's method, but in Fuller's the diazotized sulfonamide is coupled with the cheap and commonly obtainable thymol, instead of with naphthyl-ethylene-diamine. Since the coupling is done in alkaline solution, it is not necessary to add sulfamate to destroy nitrous acid. Fuller's method also has an advantage in the stability of its color; the solution after development of its color need not be protected

from ordinary daylight, and remains unchanged under usual laboratory conditions for 6 hours or more. The only relative disadvantage of Fuller's method appears to be that the optical density per unit concentration of sulfonamide is only about half as great as the density obtained with the Bratton-Marshall method; hence the samples of blood required for the Fuller method are twice as great.

b. REAGENTS.

- (1) *Stock sodium nitrite solution, 50 percent.* As described for Bratton and Marshall's method. (See par. 217.)
 - (2) *Sodium nitrite, 0.5 percent solution.* One ml of the 50 percent solution is diluted to 100 ml. This solution is prepared the day it is used.
 - (3) *Thymol in 5 N sodium hydroxide solution.* 0.5 gm of thymol in 100 ml of 5 N sodium hydroxide solution.
 - (4) *Trichloroacetic acid, 5 percent solution.* 50 gm of trichloroacetic acid per liter of aqueous solution.
- Note.* If trichloroacetic acid is not available to precipitate the blood proteins, it may be replaced by 95 percent ethyl alcohol.
- (5) *Sodium hydroxide, 1 N solution (approximate).*
 - (6) *Hydrochloric acid solutions, 1 N and 4 N (approximate).*
 - (7) *Stock standard solutions of sulfonamides.* Of each sulfa drug to be determined 100 mg is dissolved in water and the solution is diluted to 1 liter.
 - (8) *Working standards.*
 - (a) *For colorimetric analyses.* Dilute portions of 20, 10, and 5 ml of the stock standard solution of the drug to be determined to 100 ml each. The working standards contain 2, 1, and 0.5 mg of sulfonamide per 100 ml.
 - (b) *For photometric analyses.* Dilute 3 ml of the stock standard solution of the drug to be determined to 100 ml, to make a solution with 0.3 mg per 100 ml.

c. PROCEDURE FOR SULFONAMIDES IN BLOOD.

- (1) *Removal of proteins.*
 - (a) *For colorimetric analysis.* Into a 100-ml Erlenmeyer flask measure 45 ml of the 5 percent trichloroacetic acid. Then add 5 ml of blood, slowly with constant rotation of the mixture in the flask. The mixture is centrifuged, or is filtered through a dry filter. If trichloroacetic acid is not available, add 5 ml of blood with shaking to 40 ml of 95 percent alcohol and filter.
 - (b) *For photometric analysis.* In a 50-ml volumetric flask place 25 ml of the 5 percent trichloroacetic acid. Add, with shaking, 2 ml of blood. Fill to the 50-ml mark with water, mix well, and centrifuge or filter. If

trichloroacetic acid is not available, add 2 ml of blood with shaking to 43 ml of 95 percent alcohol and filter.

- (2) *Development of color to measure free sulfonamide.* Of either trichloroacetic acid filtrate (for colorimetric or photometric analysis) mix 10 ml with 1 ml of the 0.5-percent sodium nitrite solution. After 3 minutes add 2 ml of the alkaline thymol solution. The orange yellow color develops at once.

Of either alcoholic filtrate mix 9 ml with 1 ml of 2 *N* aqueous hydrochloric acid solution. Add 1 ml of the 0.5-percent sodium nitrite solution. After 3 minutes add 2 ml of the alkaline thymol solution. Wait 20 minutes for the color to develop. Then filter again.

- (3) *Development of color to measure total sulfonamide.* Ten ml of blood filtrate is heated for 1 hour with 0.5 ml of 4 *N* hydrochloric acid, and brought back to 10 ml volume, as described in paragraph 217c(3). Then 0.5 ml of 0.5-percent sodium nitrite solution is added, and after 3 minutes is followed by 2 ml of the alkaline thymol solution.
- (4) *Development of color in standards.* For colorimetric measurements a 10-ml portion of each of the three working standards is treated as described above for development of color to measure free sulfonamide in blood filtrate. For photometric measurement only the standard with 0.3 mg of sulfonamide per 100 ml is required.
- (5) *Colorimetric measurement.* Each filtrate is compared with the standard that most nearly matches it. For the standard representing 15 mg per 100 ml blood it is convenient to set the colorimeter at 10 mm; for the 10 mg standard, 15 mm; for the 5 mg standard, 20mm.
- (6) *Colorimetric calculation.*

$$\text{Mg sulfonamide per 100 ml blood} = 10.7 \times C_s \times \frac{S}{U}$$

C_s is the concentration of sulfonamide (mg per 100 ml) in the working standard, S is the reading of the standard, U the reading of the unknown. The factor 10.7 is used instead of the actual dilution, 10, because about 6.5 percent of the sulfonamide is adsorbed by the precipitate of blood proteins when the precipitation is carried out with a 1:10 dilution of the blood. The factor 10.7 corrects for the loss by adsorption.

(7) *Photometric measurement.*

- (a) *Blank.* If the reagents are pure their optical density is practically zero with light of 460 millimicrons wavelength. Hence, with such reagents, water may be used as the blank. A blank should be prepared with each new lot of reagents, however, with water in place of blood, and if the reagent blank shows a density significantly greater than that of water the reagent blank should be used, instead of a water blank.
- (b) The optical density is measured with light of wavelength 460 millimicrons. A single standard with 0.3 mg of drug per 100 ml usually suffices, because the solutions follow Beer's Law over the concentration ranges encountered.

(8) *Photometric calculations.*

$$\text{Mg sulfonamide per 100 ml blood} = 7.6 \times \frac{D_u}{D_s}.$$

D_u is the optical density of the unknown, D_s the density of the standard with 0.3 mg of sulfonamide per 100 ml. The factor 7.6 instead of 7.5 is used, in order to correct for the adsorption of about 2 percent of the sulfa drug that occurs when the blood proteins are precipitated in 1:25 dilution.

One may determine the constant, $k = \frac{7.6}{D_s}$, and use

the k in subsequent analyses.

(9) *Measurement of blood sulfonamide without colorimeter or photometer.*

- (a) In the absence of an instrument one can estimate sulfonamide, within 1 mg per 100 ml of blood, by comparison with a set of standards in test tubes. A standard equivalent to 20 mg of sulfonamide per 100 ml of blood is prepared by diluting 18.7 ml (instead of 20 ml, to correct for adsorption) of the stock standard solution to 100 ml. Of this 20 mg standard, portions of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ml are measured from a burette into a series of 15 by 150-mm test tubes, which should all be of clear glass and approximately the same diameter. To each tube add from a burette enough distilled water to make the volume up to 10 ml. The sulfonamide concentrations in the tubes are then equivalent to 2 mg per 100 ml of blood for each ml of the 20 mg standard that was added; for example, the

tube that received 4 ml of the 20 mg standard has sulfanilamide concentration equal to that of a 1:10 filtrate of blood with 8 mg per 100 ml, etc. The tubes are marked 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, indicating the mg of sulfonamide per 100 ml of blood to which each is equivalent. The color is developed in all of these tubes of graduated standards as described for development of color of free sulfonamides in blood filtrates.

- (b) The color in 1:10 blood filtrate is developed as described above to measure either free or total sulfonamide. The color of the blood filtrate is compared with that of the graduated standards. If the color of the filtrate falls midway between that of two standards, the intermediate mg per 100 ml is taken as the blood concentration.

219. Hemoglobin in Blood — Photometric

(Todd and Sandford, Clinical diagnosis by laboratory methods, 10th edition, p. 216)

a. GENERAL. Blood is diluted with 250 volumes of 0.1-percent sodium carbonate solution, forming an oxyhemoglobin solution of pH 10.7. The optical density of the solution has a maximum near the wave length 545 millimicrons, and does not change for several hours.

Two procedures will be described, one for venous blood using a 1-ml sample, and one for capillary blood using a 0.1-ml sample obtained by finger puncture. The procedure for venous blood yields exact results more easily, because less skill is required in measuring the 1-ml sample than the 0.1-ml sample. The use of capillary blood is not permissible in cases with severe shock, or with edema involving the hands. In shock the capillary blood may be 30 or 40 percent higher than the circulating blood in hemoglobin content. In cases with edema involving the hands, edema fluid from the subcutaneous tissues may contaminate the blood obtained by puncture and cause results for hemoglobin to be too low.

b. REAGENTS (sodium carbonate, 0.1 percent solution). One gram of anhydrous Na_2CO_3 is dissolved in water and the solution is diluted to 1 liter.

c. APPARATUS.

- (1) *For venous blood.* Sterile syringes, 1-ml transfer pipettes calibrated for blow-out delivery, 250-ml volumetric flasks. (If only one calibrated pipette is available, it may

be used for a series of bloods by washing it with water, alcohol, and ether after each measurement.)

- (2) *For capillary blood.* Sterile lancets, 0.1-ml pipettes calibrated "to contain," a 25-ml transfer pipette, 50-ml Erlenmeyer flasks.

d. PROCEDURE.

- (1) *For venous blood.*

- (a) Two ml or more of blood is drawn with a syringe and is transferred to a tube containing 1 to 2 mg of oxalate per ml of blood.

- (b) A 250-ml volumetric flask is filled to the mark with the 0.1-percent carbonate solution.

- (c) The blood in the tube is thoroughly mixed by stoppering the tube and inverting it 10 times, or by raising and lowering a rod with a mushroom tip 10 times through the blood.

- (d) Immediately after the mixing, a sample of 1 ml of the blood is drawn into a pipette, and is transferred to the flask with the 250 ml of carbonate solution. For accurate delivery at least 30 seconds should be taken to let the blood run out of the pipette. The drop in the tip is then blown into the carbonate solution. The 250-ml flask is stoppered, and the blood is mixed with the carbonate solution by inverting the flask 10 times.

- (2) *For capillary blood.* Pipette 25 ml of the 0.1-percent carbonate solution into a 50-ml Erlenmeyer flask. Wash the finger tip with alcohol and make certain that the skin is dry. Pierce the skin with a sterile lancet. Fill a dry, clean 0.1-ml pipette with blood from the drop by capillary attraction, holding the pipette in a horizontal position. If the blood is drawn above the mark, draw the meniscus back exactly to the mark by touching the tip of the pipette with a bit of filter paper. Make certain that no blood is clinging about the outside of the tip. Empty the pipette into the carbonate solution. Rinse the pipette 3 times by drawing carbonate solution up into the pipette and ejecting the solution back into the 50-ml flask. Mix the blood and carbonate solution by rotating the flask.

e. PHOTOMETRIC MEASUREMENT.

- (1) *Determination of k value with blood of known hemoglobin content.* In three or more samples of normal venous blood the hemoglobin is carefully determined, either by the copper sulfate gravity method (par. 215) or by the method for "total hemoglobin by carbon monoxide capacity" described on pages 341 to 345 inclusive of the

Table XXIII. Normal Concentration Ranges of Constituents Determined in Whole Blood

Constituent	Normal range
Carbon monoxide -----	0.1 to 0.3 vol. percent in nonsmokers. Up to 1 vol. percent in smokers.
Hemoglobin -----	13.7 to 18.1 gm per 100 ml. in men. 12.5 to 16.3 gm per 100 ml in women. Less in children, except infants under 6 months.
Hematocrit -----	42 to 52 ml per 100 ml blood in men. 38 to 46 in women.
Nonprotein nitrogen --	25 to 40 mg per 100 ml.
Sugar -----	80 to 120 mg per 100 ml. Blood taken before breakfast.
Urea nitrogen -----	5 to 20 mg per 100 ml.
Urea clearance -----	70 to 130 percent of average normal.

Table XXIV. Normal Concentration Ranges of Constituents Determined in Plasma or Serum

Constituent	Normal range
Albumin -----	3.4 to 5.2 gm per 100 ml.
Albumin-globin ratio (Howe method).	1.4 to 2.2.
Bilirubin -----	Less than 0.6 mg per 100 ml, all "Indirect." No "Direct."
Calcium -----	9 to 11 mg per 100 ml.
Carbon dioxide capacity.	53 to 77 vol. percent, or 24 to 34 millimoles per liter, in adults. Somewhat lower in children.
Chloride -----	98 to 106 millimoles per liter. Equivalent to 570 to 620 mg NaCl per 100 ml.
Cholesterol, total -----	130 to 330 mg per 100 ml.
Cholesterol, free -----	24 to 30 percent of total cholesterol.
Creatinine -----	1 to 2 mg per 100 ml.
Fibrinogen -----	0.2 to 0.4 gm per 100 ml.
Globulins, including fibrinogen.	2.0 to 3.5 gm per 100 ml.
Icterus index -----	Usually below 6.
Nonprotein nitrogen --	18 to 30 mg per 100 ml.
Phosphate, inorganic --	3 to 4 mg calc. as phosphorus per 100 ml, in adults. 4 to 5.5 mg in children.
Phosphatase, alkaline --	5 units or less in adults. Higher in children.
Phosphatase, acid -----	2 units or less.
Proteins, plasma -----	6.0 to 8.0 gm per 100 ml.
Proteins, serum -----	5.7 to 7.7 gm per 100 ml.
Uric acid -----	4 to 6 mg per 100 ml

1943 edition of Peters and Van Slyke's *Methods*. A 1-ml sample of the blood thus standardized is then mixed with 250 ml of carbonate solution as described in *d* above for venous blood. The optical density, D , is measured with light of wave length 545 millimicrons, the zero point of the photometer scale being set with a blank of water. The k value (pars. 151 and 157) is calculated as:

$$k = \frac{\text{Gm hemoglobin per 100 ml blood}}{D}$$

The average value of k thus obtained from the standardized bloods is used in subsequent analyses.

- (2) *Measurement of optical density of unknown.* The optical density of the 1:251 dilution of blood in carbonate solution, prepared from either venous or capillary blood, is measured with light of 545 millimicrons wavelength in cuvettes of the same size used in determining k value. Water is used for the blank.

- (3) *Calculation.*

$$\text{Gm hemoglobin per 100-ml blood} = k \times D.$$

D is the optical density of the solution, k the constant determined from standardized blood.

CHAPTER 7

GENERAL BACTERIOLOGIC METHODS

Section I. COLLECTION OF BACTERIOLOGIC SPECIMENS

220. General

Specimens of pathologic materials to be used for bacteriologic examination should be collected from locations where the suspected organisms are most likely to be found and must be handled aseptically in order to avoid contamination with extraneous microorganisms. Care must also be exercised to avoid collecting specimens at a time when the infecting organisms have probably been killed by the use of antiseptics. Closed, sterile, glass containers are required, but the type of container depends on whether the specimen can be examined immediately or must be shipped some distances. As a rule, small specimens are shipped in sealed glass tubes, protected with absorbent cotton, in tightly closed tin containers that are inclosed in larger mailing cases. Methods suitable for collecting a variety of pathologic materials from different locations are briefly indicated in the paragraphs below.

221. Materials From Eye

Collect specimens from the infected eye during the period in which the disease is progressing, as at a later time one may obtain only such saprophytes as *Corynebacterium xerose* or staphylococci. The secretions may be collected either with a cold sterile platinum loop or with a cotton swab, but one must take care to avoid contamination due to contact with the margins and angles of the lids. Sufficient material should be taken to prepare at least two stained smears in addition to the cultures. Specimens from the infected cornea, the anterior chamber, or the iris should be collected by the ophthalmologist in charge of the case. Pus from the styas should also be collected by the attending surgeon. Enucleated eyes may be sterilized externally by dipping them in boiling water or an antiseptic solution, after which they can be opened aseptically and the desired structures removed for bacteriologic examination.

222. Materials From Ear and Mastoid

In infections of the external auditory canal, the exudates may be collected on an ordinary sterile cotton swab. In otitis media or in mastoid infections, the specimens should be collected by the attending surgeon. Both smears and cultures should be prepared.

223. Materials From Nose, Sinuses, and Nasopharynx

Collect specimens from either the nasal passages or from the nasopharynx on a small sterile cotton swab, tightly wrapped on a sterile wooden applicator. Tilt the tip of the nose upward and insert the applicator gently, pushing it backward with a rotary motion until the posterior wall is reached. For meningococcus carriers surveys the West nasopharyngeal swab is a convenience. This consists of a cotton swab on a flexible wire inclosed in a bent glass tube, the latter serving to prevent contamination with mouth flora while the swab is pushed forward through the tube to the selected spot high in the nasopharynx. Material from infected sinuses should be collected by the rhinologist responsible for the case. Wire swabs may be sterilized in the large test tubes ordinarily used for bacteriological examination of water. As they are withdrawn from the mouth of the tube, they may be bent at an angle appropriate for swabbing behind the uvula. A swab moistened with broth is preferred to a dry swab. For meningococci the swab may be inserted into a tube containing about 1.0 ml of sterile horse blood or serum for transport to the laboratory. Aluminum alloy wire, 0.0625 gauge, 8 inches long may be used. One end of the wire is turned back on itself about 3/16 inch to form an attachment for the absorbent cotton.

224. Materials From Throat and Tonsils

a. Inflammatory exudates from the throat are usually collected on sterile cotton swabs slightly larger than those recommended for use in the nose. The throat should be well illuminated so that material can be taken directly from inflamed areas, which must be swabbed rather deeply to insure getting a sufficient number of the pathogenic microorganisms.

b. When searching for focal infections, or for diphtheria carriers, it may be desirable to make cultures of materials from the crypts of the tonsils. First collect material from the entire tonsillar surface on a cotton swab. Then carefully insert into the crypts a platinum loop bent at right angles. If desirable the contents of the crypts may be squeezed out by pressure or drawn out with a

special glass tube attached to a suction pump. As a rule such specimens should be collected by the attending laryngologist.

c. Tonsils removed surgically may be sterilized externally by dipping them into hot petrolatum or boiling water, or by exposure to antiseptic solutions, after which materials from inside the tonsils may be obtained aseptically and used for examination.

225. Sputum

Sputum may be collected in a sterile Petri dish, wide-mouth bottle, or vial, preferably soon after the patient awakes in the morning. The sputum is coughed up and expectorated directly into the sterile container, avoiding unnecessary introduction of saliva. The use of paper boxes or other nonsterile containers is not recommended even for specimens that are to be examined microscopically for tubercle bacilli. Specimens should be kept cold and, if possible, examined within 24 hours. If a specimen to be examined for tubercle bacilli cannot be examined within 24 hours, or is to be shipped to another laboratory, trisodium phosphate solution should be added in accordance with instructions given in par. 386. The possibility should be kept in mind that the outside of a container with sputum may be contaminated by the patient and that the tubercle bacillus is quite resistant to drying.

226. Blood

Blood for bacteriologic examination must be collected under aseptic conditions by an experienced technician or a physician. The specimens are usually collected from the median basilic or median cephalic vein. Withdraw 8 to 10 ml of blood by venipuncture. Place 2 to 3 ml of the blood into each of two flasks containing 100 ml of dextrose infusion broth or other suitable medium, and place the remainder in a large sterile test tube containing 1 ml of a 0.2-percent sterile solution of sodium citrate in physiologic salt solution. Measured amounts of the citrated blood can be placed in plates and mixed with melted agar or other media as desired. For shipment, blood is collected as above and placed in bottles of sterile dextrose broth, or, if typhoid fever is suspected, bottles of sterile bile medium may be used. Such culture bottles are labeled, packed, and shipped in double containers.

227. Blood Serum

Blood to be used for the collection of serum for agglutination, complement-fixation, and precipitation tests may be obtained with a large sterile needle. Allow from 5 to 10 ml of blood to flow di-

rectly from the needle into a sterile cotton-plugged tube. Stand the specimen in an upright position, protected from sunlight, until coagulation is complete. Loosen the clot from the side of the tube with a sterile wire or glass rod, pull out the cotton plug half way and fasten its ends to the outside of the tube with a rubber band, and centrifuge the tube until the serum is free from red cells. With a sterile pipette, transfer the clear serum to sterile vials. Cork securely, label, pack, and ship at once. Contaminated specimens deteriorate rapidly in warm weather, thus becoming unsuitable for satisfactory examination.

228. Cerebrospinal Fluid

This will be collected by the attending physician by means of a spinal tap. The fluid should be immediately taken to the laboratory where appropriate stains and cultures can be made.

229. Pleural, Pericardial, and Other Fluids From Serous Cavities

These will be collected by the attending physician by aspiration with a sterile syringe and a moderately large needle.

230. Bile

Bile collected by the surgeon at operation may be placed in a sterile tube and later inoculated into media. However, specimens are more commonly obtained by nonsurgical drainage through a sterile duodenal tube. The different fractions of bile obtained may be inoculated on duplicate sets of culture media. Such cultures may be very helpful in the isolation of typhoid bacilli from suspected cases or from carriers.

231. Feces

a. Fecal materials to be examined bacteriologically should be passed by the patient directly into a sterile container, such as a bedpan, basin, fruit jar, or Petri dish. A small portion may be collected with a sterile spatula and placed in a sterile wide-mouth bottle or vial. The specimens may be preserved by adding a small portion of feces to a vial of sterile buffered glycerol saline solution (glycerol 30 ml; NaCl 0.42 gm; K_2HPO_4 , anhydrous, 0.31 gm; KH_2PO_4 , anhydrous, 0.1 gm; distilled H_2O , 70 ml. The pH should be 7.2. This solution should be tinted pink by the addition of phenol red, and it should not be used if it becomes acid. Autoclave in vials.)

b. Specimens may be collected directly from the patient in the following manner. After cleansing the perianal skin with soap, water, and dilute ethyl alcohol, introduce into the anus a sterile cotton swab moistened with sterile broth or salt solution. To avoid contamination of the swab during passage through the anus, the swab may be inclosed in a short piece of sterile rubber tubing (8-mm bore, 1- to 2-mm wall). Just before insertion half of a 1/8-ounce veterinary gelatin capsule (disinfected by soaking in 95-percent ethyl alcohol) may be slipped over the end of the rubber tube; after insertion the capsule is dislodged by pushing the swab forward.

c. If it is desirable to collect culture materials from the lesions in ulcerative colitis, this should be done by a proctologist, and the specimen should be inoculated on blood agar and infusion broth.

232. Urine

Specimens should be collected by catheterization under aseptic conditions, but in dealing with infants, specimens must be collected directly from the cleansed urinary meatus. It should be remembered that urine samples collected by the latter method are often contaminated with colon bacilli and staphylococci. Because of the striking differential features of certain organisms, including those of the typhoid-paratyphoid group, contamination may not seriously interfere with their recognition in cultures, and specimens to be used in carrier surveys may, therefore, be passed by the patients directly into sterile containers. If done promptly, sediments from such specimens may be cultured for gonococci. Such specimens can also be used in preparing films to be examined for various organisms including gonococci, streptococci, colon bacilli, and tubercle bacilli, although the results are insufficient for anything more than a presumptive identification. In suspected tuberculosis of the urinary tract, it is safer to base the diagnosis on the examination of urine collected aseptically through a catheter and inoculated into animals. All urine specimens should be centrifuged and the sediment used for examination. The effectiveness of the centrifuging may be improved by adding sterile 2 percent sodium oxalate in amount one-tenth of the volume of the urine or other body fluids. The oxalate combines with the calcium which is always present in body fluids and slowly forms a very fine and homogeneous fog of calcium oxalate. When settling, the precipitate carries down bacteria in suspension.

233. Materials From Urethra and Prostate

a. In gonorrheal urethritis, collect pus from the urethra on a sterile cotton swab, and with this prepare two or more films. If desirable, inoculate cultures on chocolate blood agar or other special media.

b. In chronic infections, urethral and prostatic secretions may be collected subsequent to emptying the bladder. Collect this in a sterile Petri dish and use for preparing films or cultures as desired.

234. Materials From Wounds

Pus or fluid in infected wounds may be collected on sterile cotton swabs or with a platinum loop. If it is suspected that the patient has gas gangrene, this information should accompany the request in order that special examinations may be immediately made for the detection of pathogenic anaerobes. Wound specimens should be collected by the attending surgeon.

235. Autopsy Materials

Owing to the rapidity with which bacteria invade the tissues after death, all diagnostic cultures to be made on autopsy materials should be collected within 1 or 2 hours if the results are to be of maximum value. Since embalming destroys most bacteria, the cultures should be collected before this is done. In taking cultures from the various organs, it is customary to first sear the surface with a flat cautery and, after making a small incision with a sterile knife or needle, to remove sufficient material with a sterile pipette, swab, scalpel, or platinum loop.

236. Disinfection of Discarded Specimens

In many instances it is advisable to keep specimens until the bacteriologic examination has been completed, in order to have on hand sufficient material to repeat the tests if necessary. Specimens or cultures to be discarded should first be sterilized. The contaminated glassware that accumulates from day to day may be placed temporarily in a large bucket or crock filled with a 5-per-cent solution of cresol. Pipettes are usually kept in separate containers. At convenient intervals remove the glassware, place in a metal pan or pail, cover with water, and sterilize in the autoclave. Thereafter it may be safely washed and prepared again for use.

237. Data to Accompany Specimens

Each specimen sent to a laboratory should be accompanied by the appropriate form, in duplicate, signed by the requesting officer. State specifically the examination desired. Show tentative diagnosis and any other information that may be of value to the laboratory officer in selecting tests to be run. If two or more different type examinations, such as spinal fluid for a Wasserman test and for culture are desired on the same specimen, submit, if possible, separate specimens, each accompanied by appropriate request slips.

238. Precautions

Pack the specimen carefully to avoid breakage and leakage. Do not ship a liquid specimen or a specimen in a liquid medium in a cotton-plugged container. Submit a sufficient amount for all tests required, and after the specimens have been prepared, mail or deliver them to the laboratory promptly to prevent spoilage.

Section II. MICROSCOPICAL AND STAINING METHODS

239. General Remarks on Examination of Unstained Material

The examination of unstained specimens or of suspensions of living organisms may yield information that is definitely diagnostic. Such preparations are used for the detection of motility of micro-organisms, the diagnosis of spirochetal infections by dark-field examination, the diagnosis of mycotic (fungal) infections, and as a screen test for the diagnosis of bacillary (also amebic) dysentery and cholera by direct examination of stool specimens.

240. Hanging-drop Preparations

a. Place a loopful of the material to be examined (fluid medium culture or a very light suspension of growth from a solid medium) on a thin (No. 1) cover glass. Invert the cover glass over the concavity of a "well" slide, so that the droplet hangs suspended in the concavity. If the preparation is to be examined over a prolonged period, rim the cover glass or concavity with vaseline or immersion oil. Otherwise, a loopful of water may be flowed between the cover glass and the rim of the concavity to fix the preparation in position and to serve as a temporary seal.

b. In order to find the preparation under the microscope reduce the light to a minimum by means of the iris diaphragm, then locate and center the edge of the drop in the low-power field. Finally swing the high-power objective into place, find the edge

of the drop and bring it into sharp focus. At this point it will probably be necessary to readjust the iris diaphragm to admit more light. The oil-immersion objective may be used for still greater magnification.

c. Hanging drop preparations are chiefly employed to determine whether or not an organism is motile, although observation of spreading growth in semisolid motility medium (see par. 271e) is now used routinely in determining motility. True motility is a manifestation of the ability of the organism to propel itself from place to place through a fluid medium. This motion is associated with the possession of special filamentous structures, flagella, which can be made apparent only by means of special staining methods or electron-microscopy. For motility studies relatively young (5- to 18-hour) cultures should be employed. A motile organism may move sluggishly or rapidly, and the number of individual organisms in a preparation that show motility may vary widely. If one assumes that only one species of organism is represented in a suspension, the presence of even one motile cell is proof of motility. *Brownian movement* is the erratic darting to and fro of small suspended particles that results from bombardment by molecules in solution in the suspended field. It should not be confused with true motility. No progressive movement is seen, the suspended bodies move only within a small circumscribed area.

241. Moist (Cover Glass) Preparations

a. Place a loopful of broth culture on a clean slide or emulsify a very small amount of growth from a culture on a solid medium in a loopful of water, salt solution, or broth on a clean slide. Cover with a vaseline-rimmed, thin (No. 1) cover glass, and press the cover glass down to form a thin film.

b. Examine with the high-dry or oil-immersion objective. It will be necessary to reduce the light by regulating the iris diaphragm. It may be convenient to locate the edge of the cover glass, the edge of the film, or perhaps a small bubble with the low-power objective in order to get the specimen in approximate focus before swinging the higher power objective into place. It is this type of preparation that is used for dark-field microscopy.

242. Dark-field Examination

By the use of a special dark-field condenser, which gives concentrated oblique illumination, objects that otherwise are microscopically invisible can be brought into view. The dark-field condenser replaces the ordinary condenser, and the light which is

ordinarily allowed to enter through the objective is reduced by an inside metal collar or ring known as a "funnel stop." Daylight does not provide sufficient illumination, therefore, a special source of artificial light must be used.

a. ADJUSTMENT OF APPARATUS.

- (1) Remove the ordinary condenser and insert the dark-field condenser with its two lateral adjustment screws forward.
- (2) Adjust the source of light until a bright ring or spot appears on the upper surface of the condenser; the plane mirror is used.
- (3) With the low-dry objective, locate the top of the condenser and the ring etched on the surface of the condenser.
- (4) Manipulate the lateral adjustment screws until the ring is brought into the center of the field.
- (5) Remove the lower half of the oil-immersion objective, insert the funnel stock with its small end towards the lens, and reassemble the objective.

b. EXAMINATION.

- (1) Secure clean slides 1.45 to 1.55 mm in thickness, and clean cover glasses; some prefer flexible cover slips.
- (2) Rim the cover glass with a small amount of vaseline.
- (3) Place a small drop of the fluid to be examined on the center of the slide, apply the cover slip and press down to obtain a thin film, *avoiding bubbles*.
- (4) Lower the substage slightly and place a drop of thin immersion oil, *free of bubbles*, on the upper surface of the condenser.
- (5) Put the slide preparation on the mechanical stage and center the specimen.
- (6) Raise the substage until the oil is spread by contact with the slide, filling the space between the slide and the condenser.
- (7) Place a drop of immersion oil, *free of bubbles*, on the cover slip.
- (8) Lower the oil-immersion objective, and focus on the micro-organisms, which should appear as bright objects against a black background. Adjust the light for brilliant illumination, reducing, if necessary, with the condenser diaphragm.

c. SUGGESTIONS TO AVOID OR CORRECT TROUBLE.

- (1) Intense illumination is required, but it may be necessary to reduce the light by means of the diaphragm in the condenser.

- (2) Avoid air bubbles in the preparation itself and in the oil applied to the preparation.
- (3) Be sure that the funnel stop has been properly inserted in the oil-immersion objective and that it has not fallen out of position.
- (4) Finally, be sure that you have a *thin* preparation without an excess of blood, tissue elements, or debris. If too dense, the specimen may require dilution with salt solution for satisfactory results.

243. Stains and Staining Methods, General Remarks on Applications

a. GENERAL. Stained smears are used routinely in the examination of specimens and cultures in order to detect the presence of micro-organisms, to study the cellular morphology of the organisms, and to determine their staining properties. A study of cellular morphology and staining properties involves determination of the size and shape of the organism, the arrangement or grouping of individual cells (singly, in clusters, pairs, chains, packets, etc.), the possession of special anatomic structures (capsules, granules, spores, and flagella) and the reaction to special stains, such as the Gram and acid-fast stains.

b. SIMPLE STAINS. Such stains are occasionally useful for a rapid examination of material for bacteria or other cellular elements. Dyes commonly used as simple stains are methylene blue, Gram's crystal (gentian) violet, fuchsin, and safranin, the same reagents that are also used for special staining methods.

c. SPECIAL STAINS. More elaborate methods are necessary for the differentiation of micro-organisms having different staining properties and for the demonstration of special anatomic structures. Special staining methods usually involve the use of two or more reagents.

d. PREPARATION OF SMEAR.

- (1) Slides to be used for preparation of stained smears must be clean and free from a film of grease. (See par. 25.) In certain instances, as in preparation of smears of suspected tuberculosis material or for flagella stains, it is recommended that *new* slides be used to eliminate the possibility of scratched or inadequately cleaned slides that are likely to give falsely positive results.
- (2) If smears are made from cultures on a solid medium, emulsify a small amount of growth in a loopful of water on the slide and spread over an area about 1 cm in diame-

ter. If fluid media cultures are used, smear a loopful of the culture in the same manner.

- (3) Allow the smear to air-dry and fix it by passing the slide quickly through the hottest part of a Bunsen flame several times in succession; flame the underside of the slide and do not heat too strongly. The preparation is then ready to stain. Methyl alcohol or other fixatives are sometimes used in special methods. Occasionally, fixation is not required.

244. Gram Stain (Hucker Modification)

a. REAGENTS.

- (1) *Ammonium oxalate-crystal violet solution:*

Crystal violet (85 percent dye content, certified ¹)	4 gm
Ethyl alcohol (95 percent)	20 ml

Dissolve the crystal violet in the alcohol.

Ammonium oxalate	0.8 gm
Water	80.0 ml

Dissolve the ammonium oxalate in the water. Dilute the crystal violet solution 1:10 with distilled water. Mix 1 part of the diluted crystal violet solution with 4 parts of ammonium oxalate solution.

- (2) *Gram's iodine solution:*

Iodine	1 gm
Potassium iodide	2 gm

Dissolve in 5 ml of distilled water, then make-up to 240 ml with water and add 60 ml of 5 percent aqueous sodium bicarbonate. Prepare fresh solution if color loss is noted on standing.

- (3) *Counterstain:*

Safranin (2.5 percent solution in 95 percent alcohol)	10 ml
Water	100 ml

b. PROCEDURE.

- (1) Stain 1 minute with the crystal violet solution.
- (2) Wash in water.
- (3) Apply iodine solution for 1 minute.
- (4) Wash in water.
- (5) Decolorize in 95-percent alcohol for 30 seconds with gentle agitation, or until violet dye fails to appear in the alcohol. Wash in water. Acetone or equal parts acetone and alcohol may be employed as decolorizing agents, but care should be taken to wash with water as soon as the

¹ Whenever used, the word "certified" refers to certification by the Commission on Standardization of Biological Stains.

violet dye stops running, usually within a few seconds after applying the decolorizer.

- (6) Apply counterstain for 10 seconds.
- (7) Wash in water; dry without blotting.

c. APPLICATIONS AND PRINCIPLES.

- (1) The gram stain is the most valuable of the special differential stains. It is used routinely, often as the first step, in the examination of specimens and cultures. Microorganisms may be either gram-positive, gram-negative, or gram-variable. Gram-positive cells often become negative as a result of autolysis, aging, acidity of the medium and temperature of incubation.
- (2) The gram-staining property is associated with the presence of magnesium ribonucleate throughout the protoplasm. Gram's iodine acts as a mordant. Gram-positive organisms retain the violet stain on optimal treatment with 95 percent alcohol or other decolorizing agents; gram-negative organisms are decolorized and subsequently counterstained with safranin.

245. Acid-fast Stain (Ziehl-Neelsen Method)

a. REAGENTS.

- (1) *Carbol-fuchsin stain*:

Basic fuchsin (10 percent alcoholic solution)	10 ml
Phenol (5 percent aqueous solution)	100 ml

- (2) *Acid-alcohol*. Add 3 percent concentrated hydrochloric acid to 95 percent alcohol.

- (3) *Methylene blue stain*. See paragraph 247.

b. PROCEDURE.

- (1) Flood the fixed smear with carbol-fuchsin stain, and steam gently over the flame for about 3 minutes; do *not* boil.
- (2) Renew the stain as it evaporates.
- (3) Wash with water.
- (4) Decolorize by flowing the acid-alcohol over the smear until the alcohol flows colorless from the slide.
- (5) Wash with water.
- (6) Counterstain with methylene blue for 1 minute.
- (7) Wash with water, dry, and examine.

c. APPLICATIONS AND PRINCIPLES. Acid-fast stains are used for the detection and differentiation of the acid-fast bacilli, such as those causing tuberculosis and leprosy. The protoplasm of the acid-fast bacilli contains a high content of waxlike substance that makes them difficult to stain, so that concentrated stains and drastic methods (prolonged staining, usually with heat) are necessary.

Once stained, the acid-fast bacilli are much less readily decolorized than are the bacteria frequently found associated with them. The acid-fast bacteria are stained red, and the other bacteria blue.

246. Rapid Acid-Fast Stain Employing Tergitol No. 7

Cover slide with Kinyoun's carbol fuchsin (phenol, 8 gm; basic fuchsin, 4 gm; 95 percent alcohol, 20 ml; distilled water, 80 ml). One drop of Tergitol No. 7 is added and the cold stain is allowed to remain for 1 minute. Decolorize and counterstain as indicated in par. 245*b* (4) through (7).

247. Loeffler's Methylene Blue Stain (Modified)

a. REAGENTS.

Methylene blue (certified)	0.3 gm
Ethyl alcohol (95 percent)	30.0 ml

When dissolved add 100 ml of distilled water.

b. PROCEDURE.

(1) Cover the smear, prepared as usual, with the dye and stain for 1 minute.

(2) Wash with water, blot dry, and examine.

c. APPLICATIONS AND PRINCIPLES. Methylene blue is commonly used in the examination of smears and throat cultures for diphtheria bacilli. It differentiates the metachromatic granules, which stain intensely, from the cytoplasm of the cell. The dye develops polychrome properties on aging or on treatment with alkali. Certain constituents of the polychrome dye have marked affinity for the granules, which stain purple while the body of the cell stains blue. Other special stains, such as those of Neisser and Albert, may also be used.

248. Stain for Diphtheria Bacillus (Laybourn Modification of Albert Method)

a. REAGENTS.

(1) *Solution No. 1:*

Toluidin blue O	0.15 gm
Malachite green (or methyl green, Albert)	0.20 gm
Glacial acetic acid	1.00 ml
Alcohol (95 percent)	2.00 ml
Distilled water	100.00 ml

Let the mixture stand 24 hours; then filter.

(2) *Solution No. 2:*

Iodine crystals	2 gm
Potassium iodide	3 gm
Distilled water	300 gm

b. PROCEDURE.

- (1) Fix smears by heat.
- (2) Flood with solution No. 1 for 3 to 5 minutes (1 minute if methyl green is used.)
- (3) Wash with tap water.
- (4) Flood with solution No. 2 for 1 minute.
- (5) Wash, blot dry, and examine.

c. INTERPRETATION. The granules of diphtheria bacilli stain intense black, the bars dark green, and the intervening portions light green. One should bear in mind that morphology is neither adequate for identifying an organism as a diphtheria bacillus, nor for eliminating a similar organism from suspicion.

249. Moist India-ink Capsule Stain

a. REAGENT. A good grade of india ink relatively free of bacteria should be used.²

b. PROCEDURE.

- (1) Emulsify a minute amount of the material in a small loop of salt solution, broth, or water on a slide.
- (2) Add a small loop of india ink and cover with a cover slip. The fluid should spread as a very thin film.
- (3) Examine immediately with the oil-immersion objective, reducing the light with the diaphragm, until the bacterial bodies are distinctly in focus.

c. INTERPRETATION. The bacterial bodies are not stained; the capsules appearing as halos about the bacteria against a dark background. Exudates and culture fluids containing serum agglutinate the ink particles, therefore the Hiss method should be used for such materials.

250. Capsule Stain (Hiss Method)

a. REAGENTS.

- (1) *Crystal violet solution.* Make a 1 percent aqueous solution.
- (2) *Copper sulfate solution.* Make a 20 percent aqueous solution.

b. PROCEDURE.

- (1) Mix the exudate or culture into a drop of serum on a slide and smear.
- (2) Air-dry, and fix as usual.

² Not all india inks are suitable. Higgin's and Weber's waterproof drawing inks have been found satisfactory, but all bottles contain some bacteria. It is advisable to select a bottle that contains a minimum number of bacteria and no encapsulated forms, then add to this bottle 0.5 percent phenol as a bacteriostatic agent.

- (3) Cover smear with 1 percent aqueous crystal violet.
- (4) Steam gently over the flame for 30 seconds to 1 minute.
- (5) Wash off the stain with a 20 percent aqueous solution of copper sulfate.
- (6) Blot dry, and examine.

c. **PRINCIPLES.** Capsules do not stain by ordinary methods because they are composed of polysaccharides that have no affinity for the dyes. Capsules may be shown in relief with india ink or may be actually stained after impregnation with serum, according to the Hiss method.

251. Spore Stain (Dorner Method)

a. REAGENTS.

- (1) *Carbol-fuchsin stain.* See paragraph 245.
- (2) *Ethyl alcohol* (95 percent).
- (3) *Loeffler's methylene blue stain (modified).* See paragraph 247.

b. PROCEDURE.

- (1) Prepare smears and fix in the usual manner.
- (2) Stain with carbol-fuchsin stain, as in paragraph 245.
- (3) Wash in hot tap water.
- (4) Rinse rapidly with 95 percent alcohol.
- (5) Apply Loeffler's methylene blue stain for 2 to 5 minutes.
- (6) Wash, then blot dry.

c. **INTERPRETATION.** Spores are red, and cell bodies blue.

252. Flagella Stain (Leifson Method, Modified)

a. **REAGENT.** The dye powder is composed of 1.0 part by weight of basic fuchsin (calculated on basis of actual dye content), 1.5 parts of sodium chloride, and 2.5 parts of tannic acid. The staining solution is made by dissolving 1.7 gm of the dye powder in a mixture of 65 ml of distilled water and 35 ml of 95 percent ethyl alcohol. It is best to suspend the powder in the water before adding the alcohol. The stain will keep for several weeks in a tightly stoppered bottle.

b. PROCEDURE.

- (1) Prepare a slightly turbid suspension of the bacteria in distilled water, either by using a small loop of growth from an agar slant or by using the washed sediment from a broth culture. Broth cultures, as such, are unsatisfactory.
- (2) Allow a large loopful of the bacterial suspension to run down over the surface of a perfectly clean slide and set the slide aside to dry.

- (3) Put sufficient stain on the slide so that it remains heaped up during staining. Do not add more stain.
- (4) Stain for 10 minutes, or until a distinct iridescent film forms over the surface of the staining solution.
- (5) Wash with water, and if desired, counterstain for 5 to 10 minutes with a dilute aqueous solution of borax (sodium borate) and methylene blue (methylene blue 0.1 percent, borax 1.0 percent). The counterstain is not necessary.
- (6) Wash, blot dry, and examine.

c. APPLICATIONS AND PRINCIPLES. Flagella are extremely slender, fragile, presumably spiral filaments. The type of flagellation (number and position of flagella) is a species character. Flagella may be polar (single or multiple), peritrichous (multiple peripheral), or lophotrichous (polar tufts). Staining methods involve the depositing of sufficient dye in colloidal or particulate form on the filaments to make them visible. The necessity for using scrupulously clean slides and suspensions free of organic matter other than the organisms (distilled water suspensions) is emphasized in flagella staining.

253. Nigrosin Relief Stain (For Spirochetes and for General Use)

a. REAGENT.

Nigrosin (water soluble) -----	10 gm
Distilled water -----	90 ml

Boil the ingredients in a flask for 30 minutes. Add, as a preservative, 0.5 ml of formalin solution, U.S.P. Filter twice through double filter paper, and store in small, sealed test tubes.

b. PROCEDURE. A loopful of fresh exudate or culture fluid is mixed on a slide with a loopful of the nigrosin solution, then spread over the slide and dried.

c. INTERPRETATION. Micro-organisms are not stained but appear in relief as unstained bodies on a dark background.

254. India-ink Relief Stain (For Spirochetes and Capsules)

a. REAGENT. Use an india ink of good quality. (See par. 249.)

b. PROCEDURE. Stain according to the directions given for the nigrosin relief stain. (See par. 253.)

Section III. CULTURAL METHODS

255. Cultural Characteristics

Under favorable conditions of food, moisture, and temperature, an organism multiplies to become, after many generations, a mass

of many similar organisms, visible to the naked eye. In cultures in fluid media one should look for turbidity, sediment, and surface pellicle. Sometimes there is developed a color, odor, or appearance peculiar to the species. In solid media, such as agar, dispersion of the new growth is impossible; the organisms pile up into a mass (colony) the size, shape, color, or consistence of which may be characteristic of the species. The differences in growth on different media, the temperature at which it grows best, and the requirements regarding the presence or absence of oxygen are also important characteristics of each kind of micro-organism.

256. Transfer of Cultures

a. Inoculation or transfer is usually accomplished with a wire needle (platinum or nichrome) supported in a holder, the wire end being either straight or bent in a loop. This wire must be sterilized before and after use by heating to red heat in an open flame. Occasionally a sterile cotton swab or pipette may be used for transfer of inoculum. Every effort must be used to make the transfer without risk of contamination.

b. If, after inoculating, a fair amount of material remains on the needle or loop, the wire should be heated *gradually* to redness; otherwise, it is likely to scatter viable organisms over the operator and the desk.

257. Transfer From Test Tube to Test Tube

Both tubes should be held in one hand and in a slanting position to prevent dust from dropping into them. The withdrawn cotton plugs should be held between the fingers of the other hand in such a position that the ends which are to be replaced into the tubes are not contaminated by touching anything. Proceed as follows:

- a. Pass the open mouths of both test tubes through the flame.
- b. Sterilize the inoculating wire (needle or loop) by heating it red hot in the flame.
- c. Insert the wire into tube No. 1 to obtain the inoculum.
- d. Insert the wire into tube No. 2 to transfer the inoculum.
 - (1) Liquid media receive a full loopful of inoculum.
 - (2) In stab cultures, the agar is stabbed to the bottom of the test tube with the straight wire needle.
 - (3) Slanted media are inoculated by stroking the inoculum over the surface with the needle or loop.
- e. Sterilize the wire.
- f. Pass the open mouths of both tubes through the flame.

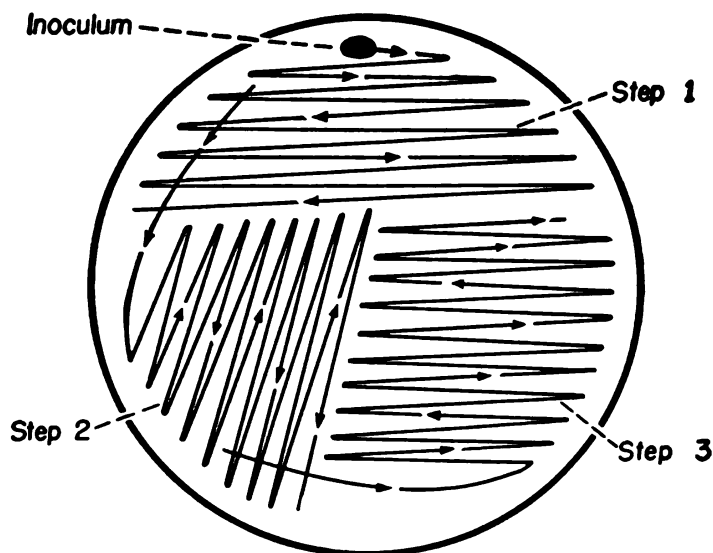
- g. Replug the tubes.
- h. Label the tubes with identification and date.

258. Streaked-plate Method

Various procedures may be used to obtain an organism in pure culture. The use of the streaked plate provides a practical method for routine use. The loop should be bent at an obtuse angle so that it will ride flat on the surface of the agar. Since the number of organisms in the inoculum may be large or relatively small, the method of streaking must be such that part of the plate is inoculated *heavily* and the remainder progressively more *lightly*. Moreover, as much as possible of the agar surface should be utilized for the best results. The following method is suggested:

- a. Collect the inoculum (specimen or culture) in a sterile loop.
- b. Distribute the inoculum over the surface of the agar in such a manner as to obtain well-isolated colonies. (See fig. 23.)

- (1) Beginning at one edge of the dish dilute out the inoculum by streaking back and forth over the same area several times, progressing out across the agar surface, until approximately one-quarter of the surface has been covered.
- (2) Flame the loop.
- (3) Streak at right angles to the originally inoculated area, carrying the inoculum out from the streaked onto the sterile surface with only the first stroke of the wire, and covering half the remainder of the sterile agar surface.



Note: The loop is sterilized between steps 1 and 2
and between steps 2 and 3.

Figure 23. Progressive steps in making a streaked plate.

- (4) Flame the loop.
- (5) Repeat as described under (3) above, covering the remainder of the sterile agar surface.
- (6) Two to four different undiluted plants may be made on separate sectors of a single plate if caution is practiced not to permit the inoculation line to cross the plate sector line. This method is frequently used to obtain massive growth of known pure cultures when well-isolated colonies are not required.
- (7) When selective media are used, e.g., SS agar, the plates should be inoculated heavily to increase the chances of isolating suspected pathogens.

259. Poured-plate Method

Another method for securing isolated colonies is by making poured plates. Proceed as follows:

- a. Melt a tube of agar and cool it to about 45° C. in a water bath.
- b. Inoculate the tube as directed in paragraph 257.
- c. Pour the inoculated agar into a sterile Petri dish, agitate the agar by moving the plate circularly on a flat horizontal surface, and cool (leave the cover partially off for a few minutes to permit water vapor to escape).
- d. For individual colony study, three such tubes may be inoculated in series and each poured as above, or a suitable dilution of the parent culture may be made in a tube of sterile broth, salt solution, or water.

260. Shake-agar Method

See paragraph 268*b*.

261. Streaked-poured-plate Method

This method is useful for the detection and study of hemolysis. It is also excellent for the culturing of swabs because it avoids the uncertainty of making proper dilutions and requires only one blood agar plate; both surface and deep colonies will appear.

- a. Streak infusion agar plates by the method described above. (See par. 258.)
- b. Pour into the plate sufficient (approx. 5 ml) 5 percent defibrinated blood agar to cover the surface of the inoculated agar.

- c. Allow the agar to solidify, and incubate in the inverted position.
- d. Examine for hemolysis after 24 to 48 hours incubation.

262. Colony Picking

Having separated individual colonies on a plate, a pure culture may be obtained by the following steps:

- a. Observe a colony with a hand lens or under the low power of the microscope and be assured that it is but one colony, well separated from adjacent colonies.
- b. Ring and number the colony with a wax pencil on the bottom of plate.
- c. Sterilize a straight inoculating wire.
- d. Touch the tip of the needle to the colony, avoiding any other colony.
- e. Inoculate selected culture media.

263. Incubation

Cultures are placed for growth in incubators that maintain a constant temperature. Most pathogenic bacteria grow well at a temperature of 37° C., but a temperature of 35° to 36° C. is considered preferable. Many saprophytic bacteria and fungi grow best at lower temperatures, and may be incubated at 20° to 30° C. Cultures in gelatin must be kept below the melting point of that medium (25° C.) either in an incubator at 20° C. or at cool room temperature, or the gelatin culture may be incubated at 37° C. and later chilled to determine whether the medium will again solidify. The length of time for incubation required for different species of bacteria varies from 24 hours to several weeks.

264. Increased Atmospheric Carbon Dioxide

Many organisms, including pneumococci, streptococci, all species of *Brucella*, and the pathogenic species of *Neisseria* grow best on primary culture in an atmosphere containing 1 to 10 percent carbon dioxide, in fact, such a condition is essential for the primary growth of *Brucella*. This may be provided in one of two ways:

- a. Place culture plates or tubes in an anaerobic jar and replace 10 percent of the atmosphere in the jar with carbon dioxide from a cylinder by first evacuating the jar to 150 mm of mercury negative pressure and then adding CO₂ to 75 mm negative pressure and air to atmospheric pressure. If vacuum or faucet suction pumps are not available, the following method may be employed.

Estimate the capacity of a jar (museum type), with air-tight lid, about 12 cm in diameter and 20 cm high. Calculate the amounts of reagents required to produce 10 percent carbon dioxide, on the basis of 0.24 gm sodium carbonate (Na_2CO_3) and 4 ml 10 percent sulfuric acid (H_2SO_4) for each 1,000 ml capacity. Place the culture plates or tubes in the jar. Place the reagents in a small, open container in jar. When the reaction begins to subside, place the lid on the jar and incubate.

b. A simpler method of securing an increased amount of atmospheric carbon dioxide is to place a small piece of lighted candle or cotton wool in an open dish or tin can lid inside the jar. After the jar is closed the burning candle will be extinguished when about 2 to 3 percent of carbon dioxide has accumulated. Since carbon dioxide is heavier than air, it accumulates at the bottom of the jar and the candle may be extinguished too soon if it is placed on the bottom. One petri dish near the top of a stack may be pulled out part-way to use as a ledge for the candle.

265. Oxygen Requirements

Bacteria differ in their oxygen requirements.

a. Aerobes grow in the average atmosphere and therefore require no special provisions. Most pathogenic bacteria are aerobes or facultative anaerobes (defined below).

b. Micro-aerophiles require an atmosphere containing a reduced oxygen content.

c. Anaerobes require a relative or absolute absence of oxygen, and special provisions for the removal and exclusion of oxygen from the cultures is required.

- (1) Obligate anaerobes require practically complete absence of oxygen.
- (2) Facultative anaerobes will grow either aerobically or under strictly anaerobic conditions.

266. Principles of Anaerobic Culture

a. OXYGEN EXPULSION. The medium is heated in vigorously boiling water for 10 minutes to drive off the dissolved oxygen, and is then cooled rapidly by immersion in cold water. A long inoculating wire or capillary pipette is used for inoculation. If the medium is agar, it should be inoculated when it reaches a temperature of 42° to 45° C. and then cooled further.

Some anaerobes, such as *Clostridium perfringens*, one of the least obligate of the pathogenic anaerobes, will grow in the lower

portions of media so prepared. However, this method is usually used in combination with one or more of the other methods.

b. OXYGEN EXCLUSION. Oxygen is expelled from the medium by boiling (as above) and further access of oxygen from the air is prevented by overlaying the surface of the medium with a seal of sterile petrolatum. (Petrolatum must be sterilized in the hot-air oven.)

c. OXYGEN EXHAUSTION. After placing the inoculated media in an airtight Novy jar or desiccator, the air in the container is exhausted by means of a vacuum pump. This method alone is not satisfactory, but is usually used in conjunction with other methods, especially that of oxygen replacement. (See *d* below.)

d. OXYGEN REPLACEMENT. The culture is placed in a Novy or similar jar, and the atmospheric oxygen is replaced with hydrogen or some other noninjurious gas by connecting the gas supply to the intake of the jar and allowing the gas to flow for about 10 minutes, after which the stopcocks are closed and the jar is placed in the incubator.

e. OXYGEN ABSORPTION (CHEMICAL AND BIOLOGICAL).

- (1) The culture tubes or plates are placed inside a desiccator or jar in the bottom of which has been placed 1 gm of pyrogalllic acid for each 100 ml of air space. To the pyrogalllic acid is rapidly added a 10 percent aqueous solution of sodium or potassium hydroxide — 10 ml for each gram of pyrogalllic acid — and the jar is quickly sealed. Many modifications of this method are used, such as placing a small culture tube inside a larger tube containing the reagent.
- (2) A carbon dioxide free atmosphere is produced by strong alkali such as used in (1) and some anaerobes may fail to grow because of that deficiency. The Rosenthal method avoids this objection. A jar with a gas outlet in the lid and which can be sealed is necessary. A desiccator is satisfactory. Place in the bottom of the jar 100 ml of 16 percent sulfuric acid per liter capacity of the jar. Place the cultures in the jar and add 5 gm of chromium metal per liter capacity and seal the lid but leave the gas outlet open until effervescence has almost ceased. Close the gas outlet and incubate. On opening the jar avoid open flames because of the hydrogen formed.
- (3) Small amounts of agar may be added to broth media to reduce the oxidation-reduction potential and permit anaerobes to grow. (See par. 268*e* and 271*e*.)

- (4) Sodium thioglycollate effectively reduces the oxidation-reduction potential and is now widely used in anaerobic culture media. (See par. 268e.)
- (5) Aerobic or facultative organisms may be grown in association with anaerobes to promote growth of the latter by using free oxygen. (See par. 268e.)
- (6) The "oat jar" is another simple method of increasing carbon dioxide and lowering oxygen. Place a handful of oats in a quart jar (e.g., desiccator). Moisten the oats and seal the jar and place it in the incubator. The sprouting oats give off carbon dioxide and absorb oxygen.

f. OXYGEN CONSUMPTION (CATALYTIC). The cultures are placed in a specially prepared jar, the lid of which has been equipped with a palladinized asbestos spool or pad. Hydrogen is passed into the jar and removes the oxygen present by combining with it to form water. The many modifications of this method, which actually makes use of several of the other principles, are widely used and are recommended where a high degree of anaerobiosis is required. (See pars. 268f and 269.)

g. SPORE CONCENTRATION. An almost universally applicable concentration method for the isolation of spore-forming bacteria, anaerobic or aerobic, is to heat the material (assumed to contain spores) sufficiently to destroy the vegetative cells. It should be remembered that spore formers do not always sporulate profusely and, under certain conditions, produce no spores. The heat treatment should be such that most, if not all, mature spores survive. There is evidence that all spores may not be so highly heat resistant as is generally believed. Therefore, the method of heating at 80° C. for 10 to 15 minutes is modified as follows:

- (1) Prepare a relatively heavy suspension of the material to be cultured.
- (2) Heat in a water bath at 65° C. for 1 hour.
- (3) Cool rapidly and culture by aerobic and anaerobic methods.

267. Media for Anaerobic Cultures

a. ROUTINE MEDIA. Practically any of the usual routine media can be used for the isolation and study of anaerobes, provided the cultures are handled in such a way that the oxygen is removed and excluded from the medium and the surrounding atmosphere.

b. SPECIAL MEDIA. Media devised primarily for anaerobic cultures include Brewer's fluid or solid thioglycollate medium and

cooked-meat medium, both of which are described in paragraph 272*q*.

c. CULTURE OF ANAEROBES. Tissue removed aseptically from a laboratory animal and transferred to a deep (10 cm) column of medium also provides conditions suitable for the culture of anaerobes.

268. Methods of Cultivation and Isolation of Anaerobes

a. LIQUID MEDIA SEALED WITH VASELINE. A layer of sterile vaseline may be used as a seal to exclude oxygen from deaerated liquid media.

- (1) Boil the medium for 10 minutes in a water bath to remove dissolved oxygen and cool by immersion in cold water without agitation.
- (2) Pipette melted sterile vaseline onto the surface of the medium to form a half-inch layer.
- (3) The following method may be used for inoculation and for subsequent examination of the culture. Warm the sides of the tube with a flame until the vaseline plug melts. Place the tube in a slanted position until the vaseline resolidifies in a thin layer, which is easily broken with the inoculating wire or pipette. To reseal, melt the vaseline again (as above) and stand the tube upright.

b. SHAKE-AGAR METHOD. Deep columns of solid or semisolid agar are anaerobic in the deep layers. The medium should be boiled for 10 minutes and cooled rapidly to 45° C. without agitation, immediately before use. These tubes are inoculated in series for dilutions of culture while the temperature of the agar is about 45° C., rotated (but not excessively) to distribute the inoculum, and then quickly cooled by immersion in cold water. The columns of agar may be sealed by adding 1 or 2 cm of sterile melted agar.

c. VEILLON TUBE METHOD. This method involves the preparation of serial dilutions in recently boiled glucose infusion agar, as described above. Portions of the medium from each tube, after inoculation, are drawn up into separate sterile lengths of small-bore glass tubing, in which the agar is allowed to solidify. Isolations from individual colonies are readily made, after incubation, by breaking the tube at points where the colonies develop.

d. USE OF PYROGALLIC ACID AND ALKALI. The quantities of pyrogalllic acid required to remove all oxygen from 100 ml of air may be calculated on the basis of 1 gm of pyrogalllic acid and 10 ml of 10-percent aqueous solution of alkali (sodium carbonate or sodium or potassium hydroxide). (See par. 266*e* for limitations to the use of pyrogalllic acid.)

(1) *Wright tube method.*

- (a) Inoculate a glucose infusion agar slant, or a series of slants in succession, if the method is to be used for isolation.
 - (b) Cut off the top of the plug (*nonabsorbent cotton*) and flame.
 - (c) Push the plug down to within less than 1 cm from the top of the slant, and insert a 1-cm plug of *absorbent cotton*.
 - (d) Pack 1 cm of pyrogallic acid crystals on top of the absorbent cotton plug.
 - (e) With a tight-fitting rubber stopper ready for immediate use, pipette 0.5 ml of alkali (sodium carbonate or sodium or potassium hydroxide) onto the pyrogallic acid.
 - (f) Plug *immediately and tightly* and seal the rubber stopper with paraffin or a paraffin-vaseline mixture.
 - (g) Incubate inverted, with the butt of the tube up.
- (2) *Other applications of this principle.* Pyrogallic acid and alkali may be used to remove the oxygen from any container that can be adequately sealed. Desiccators, special jars or culture dishes, or rubber-stoppered bottles or tubes may be used.

e. USE OF SEMISOLID AND THIOLYCOLLATE MEDIA.

- (1) *Semisolid medium.* Small amounts of agar (0.2 to 0.4 percent) reduce the oxidation-reduction potential of broth media and permit effective growth of anaerobes. This medium is described in par. 271e and is recommended for maintaining cultures of anaerobes and for motility determinations. With added carbohydrates, it is recommended for studying fermentation reactions of anaerobes.
- (2) *Thioglycollate medium.* Sodium thioglycollate is employed to reduce the oxidation-reduction potential of media for cultivating anaerobes.
 - (a) Thioglycollate fluid medium made in accordance with the requirements of USP XIII (see par. 272q) is recommended for sterility testing and for a concentration medium in cultivating anaerobes.
 - (b) In agar plate media (see par. 272q) and with appropriate materials for removing oxygen at the surface, thioglycollate is used for plate isolation of anaerobes. The recommended procedure employs the Brewer anaerobic petri dish cover which is merely placed on the thioglycollate agar after inoculation. A narrow space between

the surface of the agar and the bottom of the cover, combined with the thioglycollate on the surface, reduces the oxidation-reduction potential adequately. This method permits rapid preparation of anaerobic cultures when only a few samples are being studied. For many plates, the anaerobic jar should be used (see *f* below). In this case, it is not necessary to add thioglycollate to the medium. Another method which may be used employs a facultative anaerobe such as *Serratia marcescens*, which is inoculated heavily on dextrose agar in the top half of a Petri dish. The bottom half contains thioglycollate agar and is inoculated with the sample. The bottom is then placed lightly on the agar of the top half. Oxygen is more or less completely removed from the enclosed space by the facultative anaerobe.

f. ANAEROBIC JAR METHOD. A variety of special jars have been devised which produce anaerobic conditions by combining oxygen of the contained air with hydrogen or illuminating gas in the presence of a catalyst (see par. 269.) The Brewer anaerobic jar is available as standard equipment. The catalyst is heated by an electrical current the wires for which are in a metal tube sealed completely from the inside of the chamber. Hydrogen or illuminating gas may be used. The jar may be adapted for use in situations where vacuum pumps or electrical current are not available. A fair degree of evacuation can be achieved by a suction pump attached to a water faucet. In the absence of electrical current, a catalyst capsule (par. 269*a*) or a small evaporating dish with finely divided palladinized asbestos (par. 269*b*) may be used as a catalyst. The Brewer jar also may be employed as a vacuum jar for culturing organisms under increased carbon dioxide tension, (see par. 264). Directions for using the jar follow:

(1) *For use with hydrogen:* HYDROGEN IS AN EXPLOSIVE GAS AND ALL PRECAUTIONS MUST BE TAKEN TO AVOID LABORATORY ACCIDENTS.

- (a) After closing and sealing with Celloseal, the jar is attached to the source of hydrogen by means of a rubber tube.
- (b) The hydrogen is admitted at a pressure of 1 to 2 pounds per square inch. No preliminary evacuation is necessary.

- (c) Using the cord furnished, connection is made directly to 110-volt AC or DC current.
 - (d) Maintain the hydrogen supply and the electrical connection for a period of 30 minutes.
 - (e) Pull out electric plug, tighten clamp on rubber tubing, disconnect it, and place the jar in the incubator.
- (2) *For use with illuminating gas.*
- (a) After properly sealing, connect parts as shown in the diagram with tubing clamp open.
 - (b) Turn three-way cock to vacuum and evacuate until difference between mercury columns is 20 centimeters.
 - (c) Turn three-way cock to gas supply and allow gas to flow into the jar.
 - (d) Using cord furnished, connection is made directly to 110-volt AC or DC current.
 - (e) Maintain the gas supply and the electrical connection for a period of 30 to 45 minutes.
 - (f) Disconnect the electric cord, tighten clamp on rubber tubing, disconnect it, and place the jar in the incubator.
- (3) *For use as evacuation-replacement jar.*
- (a) The jar is sealed and then attached, by means of a three-way cock, to a source of hydrogen (generator or cylinder) and a vacuum pump.
 - (b) The jar is evacuated to 700 mm of mercury negative pressure and refilled with hydrogen.
 - (c) Step (b) is repeated three or more times.
 - (d) Tighten clamp on rubber tubing and place the jar in the incubator.
- (4) *For use as evacuation-replacement jar with catalysts.*
- (a) The jar is prepared in the usual manner, employing Celloseal or other suitable material as the sealing agent, and is attached, by means of a three-way cock, to sources of hydrogen, carbon dioxide, and vacuum. The set-up is essentially that shown in the diagram, substituting sources of hydrogen and carbon dioxide for the gas outlet.
 - (b) The jar is evacuated as completely as possible if a water pump is used or to 760 mm of mercury negative pressure if a vacuum pump is used.
 - (c) The jar is refilled with carbon dioxide to atmospheric pressure.
 - (d) The jar is again evacuated as under step (4) (b) above.

- (e) Hydrogen is then admitted slowly until the jar is filled.
 - (f) The tubing is then clamped and the jar connected to the electric current for a period of 30 minutes. After this period, it is ready to be placed in the incubator.
 - (g) It is felt that this system is the method of choice when all the essential apparatus is available. By removing a large percentage of the atmospheric oxygen by evacuation, the risk of explosion is minimized to the point where it is practically nonexistent.
- (5) *A convenient assembly of the anaerobic jar is given in figure 24.*

269. Miscellaneous Information on Anaerobic Jars

- a. **CATALYST CAPSULES.** These may be prepared as follows:
 - (1) Place 1.4 gm of asbestos wool in a porcelain evaporating dish.
 - (2) Add 10 ml of 10 percent platinum chloride and a few drops of hydrochloric acid to aid solution and stir this into the asbestos wool with a spatula.
 - (3) Dry slowly in an incubator or hot-air oven.
 - (4) Carbonize thoroughly in a yellow flame, with frequent stirring of the wool, and when the wool is well coated with soot, heat it to glowing by using a "blue" flame to reduce the mixture.
 - (5) Place the platinized asbestos between two layers of very fine wire screen, and complete the capsule by sewing the rim of the double-layer screen with wire.
 - (6) Reactivate the capsule monthly by thoroughly carbonizing it in a yellow flame, and then heating it to a red glow.
- b. **CATALYST DISH.** Palladinized asbestos in a 2 or 3 inch evaporating dish serves as an excellent catalyst to remove the small amount of oxygen remaining in an anaerobic jar after hydrogen has replaced most of the air. Before use, it is reactivated by heating thoroughly over a bunsen flame to drive off moisture.
- c. **ANAEROBIC INDICATOR TUBE.** Whenever the jar is used for the growth of anaerobes, an indicator should be included to check the development of an anaerobic state. A good, easily utilized indicator depends on the change of methylene blue from the colored (oxidized state) to the leuco form (reduced state). The procedure recommended by Fildes is — Prepare three stock solutions:
 - (1) 6.0 ml *N*/10 NaOH diluted to 100 ml with distilled water;
 - (2) 3.0 ml 0.5 percent aqueous methylene blue diluted to 100 ml with distilled water;

- (3) 6.0 gm of dextrose in 100 ml of distilled water to which has been added a small crystal of thymol. Each time the indicator solution is needed, mix equal parts of the three solutions and boil until decolorized. Place tube in the jar and begin process of securing anaerobic condition. If anaerobic conditions are maintained, the indicator tube will remain colorless. If the blue color does return, it is a sign that the seal between jar and lid leaks or that the jar has not been satisfactorily exhausted of oxygen. In using illuminating gas or other gases in which carbon monoxide is encountered, this indicator system does not function.

d. DANGER OF EXPLOSION. The introduction of hydrogen into a jar containing air or oxygen must be done very slowly, to avoid danger of an explosion, which will occur if the catalyst becomes too hot. The flow of hydrogen should be reduced if the catalyst becomes red hot. Action of the catalyst is evidenced by the formation of water vapor within the jar and by warming of the catalyst. If the catalyst fails to function, it requires reactivation. (See par. 268f.)

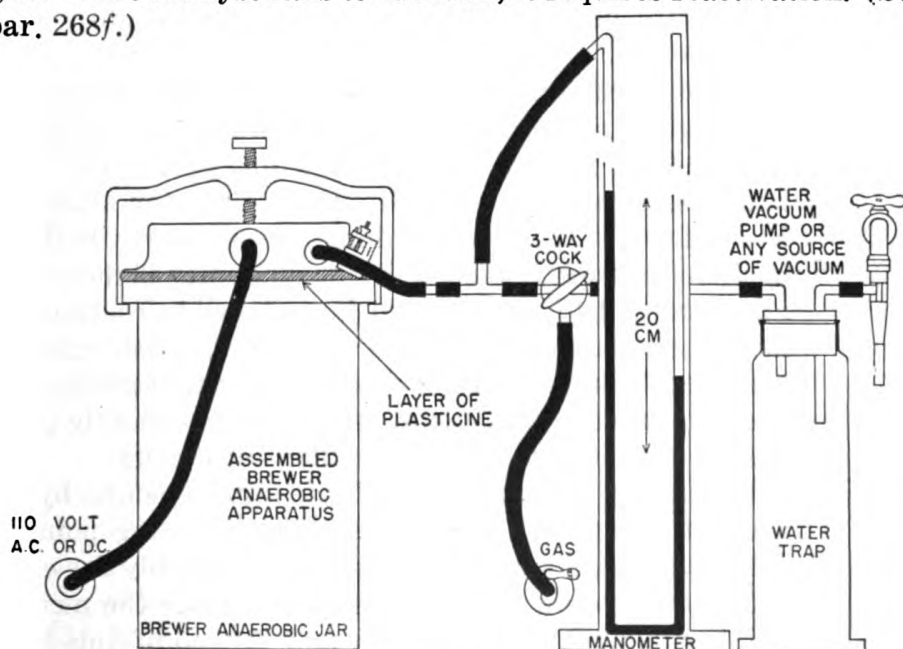


Figure 24. A convenient assembly of the anaerobic apparatus.

Section IV. GENERAL METHODS

270. General

a. PROPERTIES OF CULTURE MEDIA.

- (1) Any nutrient substance in which or upon which bacteria may grow is a culture medium. Some substances, such

as milk and potato, serve as culture media in their natural state; others are artificially compounded, such as nutrient broth and nutrient agar. The most commonly used ingredient in artificial media is "peptone." Bacteriological peptones are digestion (peptic, tryptic, or papaic) products of native proteins (meat, blood, gelatin, casein, etc.). Such peptones are not pure peptones in the chemical sense but are mixtures of proteoses, peptones, polypeptids, and amino acids in various proportions, depending on the substrates, enzymes, and manufacturing processes employed. The peptone listed in the thirteenth revision of the U. S. Pharmacopoeia is a pancreatic digest of casein. It is an all-purpose "peptone" and is especially valuable for isolating fastidious organisms such as the *Brucella* species. Enriching or inhibiting substances which are added to facilitate isolating various pathogens are indicated in paragraphs 272 and 273.

- (2) The reaction of the medium (its degree of acidity or alkalinity), measured in terms of pH, must be suitably adjusted; most bacteria prefer a neutral or slightly alkaline medium.
- (3) The buffer content of media is of importance under certain conditions. Buffer substances serve to retard changes in the pH of the growing culture, thus permitting more abundant growth before a limiting acidity or alkalinity is attained. On the other hand, in a poorly buffered medium a recognizable acidity or alkalinity is more rapidly produced. This fact is of importance in routine fermentation studies. Generally speaking, meat infusion media contain more buffer than meat extract media, and simple peptone media contain still less. Frequently phosphates are added to media as buffer substances.

Media may be liquid or solid, the latter made so by the addition of agar or gelatin. Since the melting point of gelatin is low, it can be used in solid form only for cultivation at or below room temperature. Since the melting point of agar is about 99° C., and its solidifying point approximately 39° C., it may be used for the cultivation of micro-organisms at body temperature (37° C.) or, in fact, at any temperature below its melting point. Solid media are particularly important because they enable one to separate single colonies for pure culture study from materials containing a mixture of micro-organisms, as well as to permit study of colonial characteristics. Liquid media are of limited value for isolation. They

are extensively used in studying the characteristics of pure cultures. Certain substances may be added to basic media to detect certain biochemical activities of organisms or to make the media selective by inhibiting the growth of certain organisms while permitting the growth of others.

- (4) The final step in the preparation of a culture medium is to sterilize it, that is, to kill by heat or remove by filtration all living organisms present, so that any growth following inoculation may be assumed to have originated in the inoculum. A medium so prepared and sterilized will retain its serviceability until it becomes too dry for use. Unless hermetically sealed, media should be stored in a refrigerator.

b. CLASSIFICATION OF MEDIA.

- (1) For convenience media will be classified according to the purposes for which they are ordinarily used.

Basic media contain peptone, peptone and meat extract, or peptone and meat infusion, usually with the addition of 0.5 percent sodium chloride. Agar is added when solid media are required. Such media supply the nutrients required by most bacteria.

- (2) Isolation media are used to obtain pure cultures of significant organisms, usually pathogens, separating them from others that may be present in the specimen or sample. Such media frequently contain ingredients that make them differential or selective and differential. Differential media may contain a carbohydrate and an acid-base indicator, making it possible to distinguish organisms that ferment the carbohydrate from those that do not. Selective media contain substances, such as dyes, bile salts, or other chemical substances that inhibit the growth of certain organisms and permit the growth of those that one is attempting to isolate. For most isolation purposes a medium that is selective and differential is superior to one that is merely differential.
- (3) Enriched media are usually prepared by adding blood, serum, ascitic fluid, or other special nutrient material (as indicated below) to basic media for the isolation and cultivation of organisms that are fastidious in their nutrient requirements. Usually such substances are added aseptically.
- (4) Biochemical media are used as an aid in the identification of organisms after they have been isolated in pure culture. In such media the organisms exhibit certain bio-

chemical activities that sometimes can only be detected by the employment of test reagents.

- (5) Special media may be required for certain procedures. For instance, certain media are specified by the American Public Health Association as "standard" for the sanitary bacteriologic analysis of water and dairy products.

c. **CLARIFICATION.** Clarification of the medium is sometimes required. Usually this can be accomplished by filtration through filter paper or cotton. For the removal of very finely divided particles egg white may be employed. Mix the white of an egg in a teacupful of the cool medium. Stir this into the balance of the medium. Heat slowly until the egg white has coagulated and then filter through paper or cotton.

d. **ADJUSTMENT OF HYDROGEN-ION CONCENTRATION.** The hydrogen-ion concentration of media may be determined and adjusted by electrometric or colorimetric methods. A simple, rapid, and satisfactory colorimetric method employs Hydrion test papers. This is satisfactory for adjusting the hydrogen-ion concentration of many media. The colorimetric method which has been most frequently used in media preparation is given below.

- (1) *Equipment.* A set of color standards is required. Color standard sets may be prepared for various indicators, such as phenol red, with a color range from pH 6.8 to 8.4, or brom thymol blue, with a range from pH 6.0 to 7.6. (See par. 122.) The set of phenol red standards, which is the one generally used, consists of nine tubes covering the pH scale from 6.8 (yellow) to 8.4 (red), the interval from tube to tube being 0.2. In an emergency, accurate color standards can be prepared by the Gillespie method, described in *Manual of Pure Culture Study*, issued by the Society of American Bacteriologists. The latter method requires the use of a comparator block with six holes since each color standard consists of paired tubes. Commercial comparators using colored discs are available.

- (2) *Technic.*

- (a) Select the standard tube of the desired pH (most culture media are adjusted to pH 7.2 to 7.6) and place it in the right front hole of the comparator block.
- (b) Place immediately behind it a tube of medium to which no indicator has been added. (The medium is occasionally diluted, using 1 part medium with 9 parts of distilled water. This dilutes the normal color of the medium, which sometimes interferes with color comparison.)

- (c) Place a tube containing 10 ml of the medium to which has been added 0.5 ml of a 0.02 percent phenol red solution in the left front hole.
- (d) Place immediately behind it a tube of distilled water.
- (e) Hold the comparator block toward the daylight and determine whether the medium plus indicator and the water has the same color as the pH standard and the medium without indicator.
- (f) If the medium is acid, as most freshly prepared media are, add measured quantities of *N/10* sodium hydroxide to a 10-ml portion of the medium until the color matches that of the standard tube. If the medium is alkaline, add measured quantities of *N/10* hydrochloric acid until the medium matches the standard tube.
- (g) From the quantity of *N/10* sodium hydroxide (or hydrochloric acid) used to adjust 10 ml of the medium to the desired pH, the amount of *N/1* sodium hydroxide (or hydrochloric acid) required for 1,000 ml may be determined by multiplying the number of milliliters of *N/10* solution by 10.
- (h) After the addition of sodium hydroxide (or hydrochloric acid) to the entire lot of medium and thorough mixing, recheck the pH and readjust if necessary.

e. **STERILIZATION.** After the medium has been distributed into tubes, bottles, or flasks, it must be sterilized. Usually an autoclave is used with a steam pressure of 15 pounds (121° C.) for 15 to 30 minutes, depending on the volume of medium in the containers. Note the desirability of using sterilizing indicators (see par. 27). Flowing steam at atmospheric pressure (100° C.) may be used for media containing ingredients such as sugars which may be affected by the autoclave temperatures (see par. 28). In sterilizing carbohydrates for fermentation tests, better results are obtained as follows: Filter 5 or 10 percent solutions of the carbohydrate through a bacteriological filter, store in screw-capped tubes, and add aseptically to sterile base media to give concentrations of 0.5 to 1 percent. The use of an inspissator is discussed in par. 29. Before use, any media not autoclaved in the container in which it is to be used, or sterilized by other methods, should be incubated to detect possible contamination.

f. **STORAGE.**

- (1) After sterilizing and labeling, media in cotton stoppered tubes or flasks should be stored in a refrigerator or cold room to retard evaporation and to prevent undue exposure to light.

- (2) Screw-capped bottles and tubes are available and are very useful for storing media. With these materials, most media need not be refrigerated. The caps should not be tightened until the contents have been autoclaved and cooled.

g. **DEHYDRATED MEDIA.** Media in dry (powder) form are obtainable and may be used for the preparation of many of the media described in this section. They are mixtures of the dry ingredients or, sometimes, are prepared by evaporating the prepared media to dryness (dehydrated). Such media are accurately prepared by reliable commercial manufacturers. In the dry state, they are stable and keep well at room temperature if tightly stoppered to exclude moisture.

h. **NEUTRALIZING OR ANTIBACTERIOSTATIC SUBSTANCES.**

- (1) *Para-aminobenzoic acid.* This chemical neutralizes the bacteriostatic effects of sulfonamides in the culture media. Any medium that receives initial inoculation with a sulfonamide-containing material may require the addition of 0.0002 percent of para-aminobenzoic acid. If para-aminobenzoic acid is not available, a similar concentration of procaine hydrochloride may be used. This particularly pertains to urine, blood, and to fecal cultures from patients who may have received sulfonamide therapy.
- (2) *Sodium thioglycollate.* This chemical incorporated in media not only permits the growth of anaerobic bacteria (because of its reducing action) but also neutralizes the bacteriostatic effects of mercurial and silver disinfectants. It is therefore, very useful in media used for testing such disinfectants. Since mercurial compounds are often used as preservatives in plasma, serum, biologicals, and pharmaceuticals intended for parenteral injection, thioglycollate media should be used for testing the sterility of such products.
- (3) *Antibiotics inhibitors.* Substances which neutralize the activity of some antibiotics are now available. They are used in culturing material which is apt to contain the corresponding antibiotics; e.g., penicillinase (Bacto-Penase) is used to inactivate penicillin. Bacto-Penase is also somewhat inactivating for streptomycin. In culturing blood or other body fluids containing penicillin, it is recommended that 1 ml of Penase be added to each 100 ml or less of sterile medium. This amount will inactivate 50,000 units of penicillin. It will assure inactivation of the maximum amount that would be present in 10 ml of

blood or other body fluids and also will inactivate up to 1,000 units of streptomycin. The penicillinase does not interfere with the sulfa-inactivating action of para-aminobenzoic acid which may also be added to the medium (see (1) above). Penicillinase should be kept refrigerated.

i. SELECTIVE BACTERIOSTATIC SUBSTANCES.

- (1) *Dyes*. When incorporated in media many dyes have selective bacteriostatic effects, generally more active against gram-positive bacteria. Crystal violet, brilliant green, and basic fuchsin are thus employed in well-known selective media. Thionin and basic fuchsin are used in differentiating species of *Brucella*. When isolation of streptococci is attempted in the presence of staphylococci, the latter are effectively inhibited if 1-500,000 crystal violet is contained in broth or agar media (1.0 ml of autoclaved aqueous 1-25,000 solution for each 20 ml of media).
- (2) *Sodium desoxycholate and other bile salts*. In proper combination with other substances in media the bile salts inhibit the growth of gram-positive bacteria and so are useful in the isolation of gram-negative bacteria from material in which both gram-positive and gram-negative bacteria are present. On desoxycholate agar the motility and flagellation of all bacteria are temporarily suppressed and the swarming of species of *Proteus* is considerably inhibited. If citrates are also present, as in desoxycholate-citrate agar or SS agar, the bacteriostatic effect extends to some of the gram-negative bacteria, notably the coliform bacilli. Bile salts may not be used successfully in media for the isolation of the gonococcus, meningococcus, or species of *Hemophilus*.
- (3) *Potassium tellurite*. This substance, when added to culture media to make a final concentration of 0.01 percent, inhibits the growth of most gram-negative bacteria but permits the growth of streptococci and other gram-positive organisms. If 0.03 percent of tellurite is used, most streptococci are inhibited but staphylococci and corynebacteria are still able to grow. Potassium tellurite is useful not only in agar for the isolation of *Corynebacterium diphtheriae* but also may be added to broth when one is trying to isolate gram-positive bacteria, notably streptococci, from mixed culture material overgrown by species of *Proteus* or other rapidly growing gram-negative bacilli. For this purpose add to a 5-ml tube of blood

or serum broth, 0.25 ml of a sterile (autoclave) 0.2 percent aqueous solution of potassium tellurite and inoculate with a loop of the mixed culture. After incubation for 12 to 18 hours, transfer to a blood agar plate.

- (4) *Chloral hydrate*. When added to nutrient agar to make a final concentration of 0.1 percent, chloral hydrate has little or no inhibitory effect on either gram-positive or gram-negative bacteria but does prevent the swarming of species of *Proteus*, rendering them temporarily non-motile. Unlike bile salts and tellurite, chloral hydrate does not make blood and so may be used in blood agar plates without interfering with the development of characteristic zones of hemolysis by streptococci and other organisms. Add 0.25 ml of sterile (autoclave) 5-percent aqueous solution of chloral hydrate to a 12-ml tube of melted agar or blood agar for plating.
- (5) *Antibiotics*. These substances are finding increasingly wider use in media employed to facilitate isolating certain organisms where the presence of others is a handicap. In paragraph 399c it will be noted that penicillin and streptomycin are added to media for isolating fungi to inhibit accompanying bacteria.
- (6) *Sodium azide*. This compound, by inhibiting growth of gram-negative organisms, is used to facilitate isolating hemolytic streptococci from the respiratory passages. Special purpose broth (par. 272i), containing added glucose (0.02 gm to 100 ml of broth) and defibrinated rabbit blood (5 ml to 100 ml of broth), is tubed in 2 ml amounts. On the day the medium is to be used, 0.15 ml of an autoclaved aqueous solution of sodium azide (1 to 1,000) and 0.1 ml of autoclaved aqueous solution of crystal violet (1 to 25,000) are added to each tube. Swabs are placed in the azide broth within 1 hour after obtaining the sample. After overnight incubation, subcultures are made on blood agar plates by streaking from the broth. The crystal violet effectively inhibits growth of staphylococci.

271. Basic Media

a. NUTRIENT BROTH (MEAT EXTRACT BROTH).

Beef extract	3 gm
Pancreatic digest of casein or peptone.....	10 gm
Sodium chloride	5 gm
Distilled water	1,000 ml

Add the weighed ingredients to distilled water and heat slowly to 65° C., stirring until dissolved. Adjust loss of volume or weight

with distilled water, and if perceptibly turbid, clarify by filtration through paper. Adjust to pH 7.2 to 7.6. Autoclave 15 minutes at 15 pounds.

b. NUTRIENT AGAR (MEAT EXTRACT AGAR).

Meat extract broth.....	1,000 ml
Agar (preferably granular).....	15 to 20 gm

Add agar to the broth and dissolve by boiling or autoclaving. Adjust pH to 7.2 to 7.6, dispense, and sterilize.

c. MEAT INFUSION BROTH.

Beef, veal, or pork finely ground and free from fat..	500 gm
Pancreatic digest of casein or peptone.....	10 gm
Sodium chloride	5 gm
Distilled water	1,000 ml

Mix the meat and the water thoroughly and infuse for 1 hour at room temperature. Boil for 5 minutes, strain through gauze, and filter through paper. Add the peptone and salt, stir until the peptone is dissolved, and adjust to pH 7.8 to 8.0. Boil for 20 minutes and bring back to original volume (1,000 ml) or weight with distilled water. Check the reaction. If necessary, readjust to pH 7.6 and boil for 5 minutes. Filter through paper. Distribute into tubes, flasks, or bottles, and sterilize.

d. MEAT INFUSION AGAR.

Meat infusion broth	1,000 ml
Agar (preferably granular)	15 to 20 gm

Add agar and proceed as in preparation of meat extract agar. Adjust to pH 7.4.

e. SEMISOLID MEDIA.

- (1) Media containing 0.2 to 0.4 percent agar are known as semisolid media. These are used chiefly for maintaining cultures, determining motility and, with added carbohydrates, for fermentation studies. Because small amounts of acids are not readily dispersed throughout the medium, positive fermentation reactions may often be determined more quickly than in liquid media. Cultures should be examined at rather frequent intervals and the cultures removed from the incubator when fermentation is noted. Prolonged incubation may cause destruction of indicators and reactions may then be difficult to determine. Anaerobes and certain other organisms (e.g., gonococci) grow better in semisolid media because of reduced oxidation-reduction potential (see par. 268e). These media, thus, are ideal for studying fermentation reactions of these organisms. (Jour. Bact. 47: 455, 1944.)
- (2) For motility studies, the surface of the medium is merely pricked with an inoculating needle containing the culture

being studied. Motility is indicated by clouding of the medium after incubation, due to the spread of motile organisms from the site of inoculation.

- (3) For fermentation, the medium (such as (a) below) should be used soon after autoclaving or it should be placed in a boiling water bath for 5 minutes to drive off dissolved oxygen, cooled rapidly, and inoculated soon after by stabbing to the bottom of the tube. No sealing material, anaerobic jars, or other special devices are necessary for cultivating clostridia in the medium given in (a) below. This medium is excellent for maintaining stock cultures of both aerobes and anaerobes. Viability is noticeably greater than in broth or agar slant cultures.
- (a) The following medium is recommended for both motility and, with 1 percent added carbohydrate, for fermentation studies:

Pancreatic digest of casein.....	2.0 gm
Agar, powdered	0.35 gm

For fermentation tests, add 0.002 gm or 1 ml of a 0.2 percent solution of phenol red. The 0.2 percent solution is prepared by dissolving 1 gm of the powdered dye in 40 ml of N/10 NaOH, then adding 460 ml of distilled water. Add the agar to 100 ml of cold water, allow to stand a few minutes, then add the other ingredients. Dissolve by boiling, cool, and adjust the pH to between 7.2 and 7.4. Tube and autoclave at 15 pounds for 15 minutes. Store at room temperature.

- (b) The following semisolid medium is used in special *Salmonella* studies for determining and enhancing motility. The medium given in (a) above is satisfactory for most routine motility determinations including *Salmonella*.

Pancreatic digest of casein.....	1.0 gm
Meat extract	0.3 gm
Gelatin, pulverized	8.0 gm
Agar, powdered	0.4 gm
Sodium cholride	0.5 gm
Distilled water	100.0 ml

Allow the gelatin to swell in cold water before adding other ingredients. Boil to dissolve, cool and adjust pH to 7.2 to 7.4. Autoclave in tubes. Inoculate the surface of the medium and incubate.

272. Isolation Media

a. EOSIN METHYLENE BLUE (EMB) AGAR (FOR TYPHOID-PARATYPHOID-DYSENTERY ISOLATIONS). Prepare as for Levine's

eosin methylene blue agar in water analysis (par. 275*g*) but reduce dye content a half by using 1 ml of 2 percent eosin and 1 ml of 0.5 percent methylene blue per 100 ml of medium.

This medium is available in dehydrated form. It differs from the media discussed in *b* and *c* below in not inhibiting coliform organisms. Gram positive organisms are inhibited, however.

b. PLATE MEDIA SOMEWHAT INHIBITORY TO COLIFORM ORGANISMS (FOR TYPHOID-PARATYPHOID-DYSENTERY ISOLATIONS). MacConkey's Agar and Desoxycholate Agar (*not* Desoxycholate-citrate) are less selective than those given in *c* below. Because of the variable resistance of some pathogens to the inhibitory agents present in the more selective media, it is recommended that either MacConkey's or Desoxycholate medium be used in conjunction with those given in *c* below when attempting to isolate enteric pathogens. Unless many specimens are being handled and time and facilities are limited, it is well also to inoculate EMB agar. These media are available in dehydrated form.

c. SPECIAL SELECTIVE MEDIA (FOR TYPHOID-PARATYPHOID-DYSENTERY ISOLATIONS).

- (1) *Salmonella-Shigella (SS) and Desoxycholate-citrate Agar.* These are available in dehydrated form. They are not autoclaved. The surfaces of the plates should be dry and the prepared media should not be used after more than a few days' storage. One or the other of these media, together with one of those given in *b* above, should be used in all routine examinations for enteric pathogens. Plates should be inoculated heavily. (Note (2) and (3) as well as *d* below.)
- (2) *Brilliant green agar (Kauffmann).* This medium is recommended where salmonella organisms (exclusive of *S. typhi*) are sought. The medium is inoculated heavily and one of the media given in *b* above also should be inoculated. This medium should not be confused with brilliant green bile agar.
- (3) *Bismuth sulfite agar.* This medium is unsurpassed for isolating typhoid bacilli. Inoculate heavily and also use one of the media given in *b* above.

d. SELENITE-F BROTH. This medium is available in dehydrated form. Although tetrathionate broth following is preferred for isolating *Salmonellae*, selenite broth is probably the medium of choice for *routine* work, since tetrathionate broth is unfavorable for *Shigellae* or the typhoid bacillus. Primary plating media are inoculated with mucous or fecal material on cotton swabs. Then the swabs, containing 0.1 to 1.0 gram of inoculum, are placed in

tubes of enrichment media and the specimen emulsified. Coliform bacteria are inhibited while enteric pathogens increase in number. Enrichment media are incubated for 16 to 18 hours and, if primary plates are negative, a large loopful of the enrichment broth culture is streaked on additional (secondary) plates. (See *b* and *c* above.)

e. TETRATHIONATE BROTH. Kauffmann's modification of this medium is considered superior for isolating *Salmonellae*, particularly when used in combination with brilliant green agar (see *c* above). It is used under the same circumstances discussed in *d* above and is inoculated and incubated in the same manner. *It should be emphasized that it is not a favorable medium for Shigellae or for S. typhi.* The dehydrated tetrathionate broth base (Difco) should be modified to conform to the Kauffmann formula as follows:

- (1) To each 100 ml of the broth base, add:

Calcium carbonate	4 gm
Sodium thiosulfate	2 gm

- (2) Sterilize at 15 lbs. pressure for 20 minutes.

- (3) Prepare iodine solution:

Iodine	20 gm
Potassium iodide	25 gm
Water	100 ml

Add only small amounts of the water until the iodine is dissolved, then add the remainder.

- (4) To 100 ml of the *cooled* broth base add:

Iodine solution	2 ml
Brilliant green, 1 to 1000.....	1 ml

- (5) Distribute medium into sterile tubes (preferably screw-capped) without further heating and store (not over 2 weeks) at room temperature.

f. ALKALINE MEDIA FOR ISOLATING VIBRIO COMMA.

- (1) *Enrichment broth.* Dissolve the following ingredients in 1 liter of warm distilled water:

Pancreatic digest of casein or peptone.....	10 gm
Sodium chloride	5 gm

Adjust the pH to about 8.6 (8.15 ml of normal NaOH generally gives the desired alkalinity). Distribute into tubes in 10 ml amounts and autoclave. A tube of this alkaline broth is inoculated with several loopfuls of watery stool or with a flake of mucus and incubated for 8 hours. Within this time vibrios will grow freely at the surface, outgrowing other intestinal bacteria.

- (2) *Plating media.* These are inoculated directly with the stool as well as with the incubated enrichment broth.

(a) Dieudonne's Alkaline Blood Agar.

Blood, beef, defibrinated	150 ml
Potassium hydroxide (normal)	150 ml
Nutrient 3 percent agar (pH 6.8)	700 ml

Mix the blood and potassium hydroxide and steam 30 minutes in an Arnold sterilizer or in an autoclave with the outlet open. Melt the agar and add it to the mixture. Pour plates and allow them to harden uncovered but protected with paper. Place strips of filter paper between each dish and cover to absorb moisture and ammonia. Incubate 15 hours at 37° C. before using.

(b) Good results have also been obtained with nutrient agar with pH adjusted to 8.4.

g. LOEFFLER'S MEDIUM (FOR *C. DIPHTHERIAE*). The value of this traditional medium for isolating the diphtheria bacillus has been seriously questioned in recent years. Its composition is variable, its growth-promoting and commensal-inhibiting properties have been overestimated and it fails to grow some strains of diphtheria bacilli. Nevertheless, the so-called typical morphology of the diphtheria bacillus is based upon its appearance when grown on this medium. Tellurite media (as given in *h* below) are being used more and more widely for direct isolation. Loeffler's medium is available in dehydrated form. It should be sterilized with the precautions discussed in *n*(5). In addition to its traditional use, this medium may be used for early determination of pigment production by many organisms and for testing the protein digesting ability of those bacteria which will grow on it.

h. TELLURITE MEDIUM (FOR *C. DIPHTHERIAE*). Agar base (see par. 272*j*). Sterilize at 15 pounds steam pressure for 15 minutes, cool to 50° C. and add the following (to each 100 ml) before pouring plates:

Aqueous potassium tellurite (1 percent)	1.0 ml
---	--------

(sterilized by filtering) and:

(1) *Either*:

Sterile human serum (pooled and sterilized by filtering)	5.0 ml
---	--------

(2) *Or*:

Sterile citrated or defibrinated blood	10.0 ml
--	---------

Caution: Avoid inhaling the potassium tellurite.

i. SPECIAL PURPOSE BROTH MEDIUM.

- (1) Broth (such as Trypticase Soy Broth) made with pancreatic digest of casein and other nutrients is recommended for blood culture and for general cultivation of fastidious organisms. It is available in dehydrated form. (Some lots of Tryptose have been found to inhibit strains of *Brucella*. (Jour. Bact. 57:1, 1949.))

- (2) The following technic is recommended for blood cultures because it precludes frequent transfers from the blood culture to plate media and thus reduces chances of contamination. It also reduces the chance of infecting laboratory personnel in the case of *Brucella* organisms. Medium containing 3 percent agar is autoclaved and allowed to solidify on the side of a bottle. Trypticase Soy Agar (*j* below) with 1.5 percent added agar and 0.1 percent added 1-cystine is recommended. Add to the bottle sterile broth such as Trypticase Soy Broth (*j* below) with 0.005 percent added para-aminobenzoic acid and 0.05 percent added sodium thioglycollate and 0.1 percent added 1-cystine. This is suitable for most organisms including aerobic, micro-aerophilic or anaerobic organisms. The para-aminobenzoic acid will neutralize sulfonamides if present. Add the blood to the broth, about 2 ml of blood for each 20 ml of broth, and incubate. Every other day tilt the bottle so as to spread the broth over the agar, and observe the agar slant for colonies.

j. SPECIAL PURPOSE AGAR MEDIUM.

- (1) An agar base (such as Trypticase Soy Agar) made with pancreatic digest of casein and other nutrients is recommended for a blood agar base, and for cultivating *Brucella* species and other fastidious organisms. It is available in dehydrated form. In addition to containing agar, it differs from the broth medium in not containing added phosphates or dextrose. (Dextrose should not be included in a blood agar base because it inhibits beta hemolysis.) Tryptose agar also may be used, but attention is directed to the discussion of Tryptose in *i* above.
- (2) For differentiating species of *Brucella* by the selective inhibition of dyes (Huddleson method), two separate media are prepared — one containing basic fuchsin and the other thionin. Add 1.0 ml of a 0.1 percent aqueous solution of basic fuchsin or thionin per 100 ml of medium, making final concentrations of 1:100,000.

k. DEXTROSE CYSTINE BLOOD AGAR (FOR *PASTURELLA TULARENSIS*). Dissolve and autoclave Trypticase Soy Agar (*j* above) or meat infusion agar. Add 0.1 percent cystine and, with occasional shaking, heat in flowing steam (autoclave with outlet open or Arnold sterilizer) for 2 hours. Cool to below 60° C., add 5 percent of sterile defibrinated rabbit or horse blood, and heat at 60° C. for 2 hours, twirling the flask occasionally to keep the blood well mixed with the agar. Add 1 percent of dextrose from a

sterile 50-percent solution. Tube aseptically, slant and incubate to test sterility.

l. POTATO GLYCEROL BLOOD AGAR (BORDET-GENGOU) (FOR HEMOPHILUS PERTUSSIS).

Freshly and finely sliced potato.....	100 gm
Glycerol (4-percent aqueous solution).....	200 ml

Mix and steam or boil until the slices of potato are soft. Strain through gauze and allow the remaining particles of potato to sediment. Draw off the supernatant glycerol extract.

Glycerol extract	50 ml
Sodium chloride, 0.6 percent aqueous.....	150 ml
Agar	5 gm

Mix thoroughly and filter through sterile gauze into a sterile the agar, and then autoclave or heat in flowing steam until the agar is dissolved. The pH is about 6.0 and no adjustment is necessary. Distribute convenient amounts into tubes, flasks, or bottles, and sterilize in the autoclave. Store until needed.

For use, melt the agar, cool to 50° C., and add 20 to 30 percent of fresh, sterile, defibrinated rabbit, human, or sheep blood. The blood should be used when fresh, never more than 72 hours after it has been obtained. Mix well by twirling the tubes or flasks to avoid bubbles, and slant in tubes or pour into Petri dishes as desired. Plates may be used as long as they remain moist and red, usually 2 or 3 weeks if kept in the refrigerator. Isolation of *H. pertussis* is significantly increased if 0.3 unit of penicillin is incorporated into each ml of medium before it is poured. The potato glycerol agar base is available in dehydrated form as Bordet-Gengou Agar Base.

m. PETRAGNANI MEDIUM (FOR MYCOBACTERIUM TUBERCULOSIS).

Potato (peeled and cut into small pieces).....	75 gm
skimmed milk	150 ml
Potato flour	6 gm
Peptone	10 gm

Mix and heat in a double boiler for 10 minutes, with frequent stirring. After the mixture becomes pasty, continue to heat for 1 hour. Add sterile distilled water to make up volume, and cool to 50° C. Add the following mixture.

Eggs (whole)	4
Egg yolk	1
Glycerol	12 ml
Malachite green (2.0 percent aqueous solution).....	10 ml

Mix thoroughly and filter through sterile gauze into a sterile distributing funnel. Distribute into tubes. Slant and inspissate for 2 hours at 70° to 75° C. on 3 successive days, or autoclave as described above under Loeffler's medium.

n. LOWENSTEIN-JENSEN MEDIUM (FOR M. TUBERCULOSIS). This medium has repeatedly been shown to be superior for isolating

tubercle bacilli. The method of preparation follows. The use of fresh eggs is recommended, although some workers have used dehydrated eggs (17 gm = 1 egg) with success.

- (1) Mix the following ingredients:

Potassium dihydrogen phosphate -----	4.0 gm
Magnesium sulfate -----	0.4 gm
Magnesium citrate -----	1.0 gm
Asparagin -----	6.0 gm
Glycerol -----	20.0 ml
Distilled water -----	1,000.0 ml

Heat to dissolve, then boil slowly for 1 hour or steam in open autoclave 2 hours; cool and place in refrigerator overnight.

- (2) To each 100 ml of cold base solution (1) above add 5 gm of potato starch. Stir well, then place in a double boiler and stir gently until a smooth solution is obtained. Allow to stand for 1 hour in a water bath at 56° C.
- (3) For each 100 ml of the starch base solution (2) above use 166 ml of *fresh* eggs. (Roughly, 1 egg = 50 ml.) Dip each egg in alcohol, allow to dry, then crack into a graduated cylinder. The eggs and starch solution are mixed by using a blender or mixing machine. During the mixing, add malachite green (0.8 ml of 2 percent aqueous solution per 100 ml of egg-starch mixture). After mixing, allow to stand for 2 or 3 hours to expel air bubbles.
- (4) Tube in screw-cap bottles.
- (5) Slanting and sterilizing can be accomplished in an autoclave if screw-capped tubes are used. It is well to wrap newspapers around the baskets of tubes. Close all the ports and the door before turning on the steam. If the exhaust of the autoclave is equipped with an automatic thermo-element type trap, it is necessary to have this line equipped with a valve which can be closed, thereby making it possible to maintain a mixture of air and steam in the autoclave during coagulation. Run the pressure as quickly as possible to 10 pounds and hold for 10 minutes to coagulate the medium. At the end of coagulation, open the lowest port (or valve in thermo-element type trap line if such is used) and replace the entrapped air with steam, maintaining constant pressure while replacing the air-steam mixture with live steam, as any appreciable change in pressure will cause the slants to be filled with bubbles and render the medium useless. Maintain steam pressure for 20 minutes at 15 pounds (121° C.). After sterilization is completed, the source of steam is cut off. All ports and the safety valve should be

tightly closed as soon as the steam is shut off, allowing the autoclave to cool slowly. Do not open any ports until the steam pressure is nil.

- (6) Screw-topped tubes may be coagulated in slanting position in an inspissator at 80° C., then autoclaved, taking precautions regarding temperature and pressure changes as before.
- (7) Some workers believe that a superior medium is obtained if sterile distilled water is used, the ingredients added as aseptically as possible and the medium heated only in an inspissator to coagulate it. It is then incubated and contaminated tubes are discarded. Excessive heating appears to liberate inhibitory substances. The medium should be refrigerated and, if not used, discarded after 2 weeks.

o. SABOURAND'S MEDIA (FOR FUNGI). This low pH and high sugar-content medium is used for isolating, identifying, and maintaining fungi. Other satisfactory media (see par. 399c) now are available or preferred for isolating fungi from sources apt to contain numerous bacterial contaminants.

Dextrose or maltose	4.0 gm
Pancreatic digest of casein or peptone.....	1.0 gm
Powdered agar	2.0 gm
Distilled water	100.0 ml

Add the peptone and agar to cold water, allow to stand several minutes, boil to dissolve the agar, add the sugar, cool, adjust pH to 5.6 and autoclave in tubes, flasks, or bottles. Do not use more than 15 pounds steam pressure to avoid hydrolyzing the agar.

p. CORN MEAL AGAR (FOR FUNGI).

Distilled water	1,500 ml
Corn meal	62 gm
Agar	19 gm

Heat water and corn meal at 60° C. for 1 hour. Filter through paper and adjust the volume to 1,500 ml. Add the agar and heat in an Arnold sterilizer for 1¼ hours. Filter through cotton, tube, and sterilize in autoclave at 15 pounds for 15 minutes. Adjustment of the reaction is not necessary.

q. THIOLYCOLLATE MEDIA (FOR ANAEROBES AND FOR STERILITY TESTS). The use of sodium thioglycollate in media is discussed in paragraph 268e.

- (1) A fluid medium for cultivating anaerobes and for sterility testing is available in dehydrated form (Thioglycollate Fluid Medium). It is made in accordance with the requirements of U.S.P. XIII and the National Institute of Health for a sterility test medium. The prepared medium should not be refrigerated. It contains an indica-

tor, resazurin, which becomes pink when oxidized. If more than 30 percent of the medium has become oxidized (i.e., has turned pink in the uppermost 30 percent) the broth may be heated *ONCE* in steam or boiling water, cooled, and used.

- (2) For a thioglycollate agar plate medium Anaerobic Agar Brewer should be used. It is available in dehydrated form.

r. ROBERTSON'S COOKED-MEAT MEDIUM (FOR ANAEROBES).

Beef heart, fresh and ground, with all fat, fascia, and blood vessels removed.....	500 gm
Pancreatic digest of casein or peptone.....	10 gm
Distilled water	1,000 ml

Mix the ingredients, and bring them to a boil. Adjust to pH 8.0. Allow the mixture to simmer 1½ hours; readjust pH. Separate the broth from the meat and place the former into flasks. Autoclave 15 minutes at 15 pounds. Place the meat on clean filter paper and dry in oven at 56° C. for 48 hours. Place the desired quantity of dried heart in a test tube and add 10 ml of the broth. Autoclave, cool, and titrate. Allowing for an acid drift, adjust the reaction so that medium will have a final pH of 7.4 to 7.6. Resterilize.

s. DEXTROSE BRAIN BROTH (FOR STREPTOCOCCI AND ANAEROBES).

Fresh calf brain	5 to 10 gm
Dextrose veal infusion broth.....	35 ml

Prepare a flask of veal infusion broth, adjust the reaction to pH 7.8, and add dextrose (1 gm per 100 ml of medium). Wash 5 to 10 pieces of calf brain, 1 ml in size, in running water and place in the bottom of a large test tube (200 × 25 mm); add 35 ml of infusion broth, autoclave at 15 pounds for 20 minutes, and cool. Remove 10 ml of the supernatant fluid and check the reaction. If the reaction is pH 7.4 to 7.6, it is satisfactory, but if there has been a greater acid drift, adjust to pH 7.6. Estimate from the titration of the 10-ml portion the amount of NaOH required to adjust the reaction for bulk of the broth. Then fill the desired number of tubes with similar quantities of the brain tissue and broth (pH adjusted, if necessary). Sterilize in the autoclave at 15 pounds for 20 minutes. Incubate at 37° C. to determine sterility.

t. BRAIN HEART INFUSION AGAR (FOR FUNGI). This medium is available in dehydrated form and should be supplemented with 1 percent glucose. Final pH 7.4 to 7.6.

u. CRYSTAL VIOLET OXGALL AGAR (FOR FUNGI). This medium is available in dehydrated form, but can also be prepared as follows:

Dextrose	1.0 gm
Pancreatic digest of casein or peptone.....	1.0 gm

Oxgall (dehydrated) -----	1.5 gm
Agar -----	2.0 gm
Distilled water -----	100.0 ml

Add 0.02 ml crystal violet solution (1.25 gm dissolved in 25 ml 95 percent ethyl alcohol) and autoclave at 10 to 12 pounds for 15 minutes. Cool to 45° C. and add 30 units of streptomycin per ml of medium except when culturing for *Nocardia* or *Actinomyces*. No pH adjustment is necessary. Dispense in plates.

v. POTATO DEXTROSE AGAR (FOR FUNGI).

Potato -----	300.0 gm
Dextrose -----	5.0 gm
Agar -----	7.5 gm
Distilled water -----	500.0 ml

Cut whole potato into 1 inch squares, steam in 500 ml distilled water for 1 hour. Strain through moist cloth, make up to volume with distilled water and add glucose and agar. Heat to dissolve, tube or flask and autoclave at 15 pounds for 20 minutes. No pH adjustment is necessary.

w. GONOCOCCUS ISOLATION MEDIA. The two media given below were selected from several which were evaluated recently (Amer. Jour. Syphilis, 33:164, 1949). These media should be used while moist and should be warmed to 37° C. if they are refrigerated before use. Overnight incubation to eliminate contaminated plates is recommended. Where treatment of the patient with penicillin is known to have occurred prior to culture, or where use of the drug by the patient is suspected, penicillinase should be added to the media before pouring plates (see par. 270h(3)).

(1) *Double strength starch agar base with hemoglobin and supplement.*

(a) Prepare a double strength agar base as follows:

Proteose peptone No. 3

or

Pancreatic digest of casein-----	30 gm
Sodium chloride -----	10 gm
Dipotassium phosphate (K ₂ HPO ₄) -----	8 gm
Potassium acid phosphate (KH ₂ PO ₄) -----	2 gm
Agar, powdered -----	20 gm
Corn starch -----	2 gm

Suspend the above in 1 liter of cold distilled water, heat to the boiling point, adjust pH to 7.2, distribute in 100 ml quantities, and autoclave. It may be stored until needed. The base is available in dehydrated form as "GC Agar Base."

(b) Prepare double strength hemoglobin in accordance with directions given in paragraph 273d(2).

(c) To prepare the medium for use, 100 ml of sterile melted double strength agar base (1) (a) above and

100 ml of sterile hemoglobin solution (1) (b) above should be adjusted to 50° to 60° C. then mixed, avoiding formation of air bubbles. After the temperature is below 50° C., 2 ml of supplement A or B (d) below are added aseptically and plates are poured.

- (d) Supplement A and B (Difco) are especially prepared thermolabile yeast concentrates containing adequate amounts of the growth-accessory factors, particularly glutamine, cocarboxylase, and coenzyme, required by the more fastidious strains of the gonococcus, as well as *H. influenzae*. Supplement A differs from B in that A also contains crystal violet which suppresses growth of many of the common contaminants.
- (2) *Agar base with hemoglobin and supplement A*
 - (a) Proteose No. 3 agar has been most widely used as the agar base. It is available in dehydrated form as a standard item. Blood agar base made with pancreatic digest of casein (j above) also may be used.
 - (b) Prepare either of these, as well as hemoglobin, in double strength according to the instructions given in paragraph 273 d(2) for chocolate agar.
 - (c) Before pouring plates (after the agar base and hemoglobin have been mixed, add 2 ml of supplement A or B (A preferred) under aseptic conditions to the 200 ml mixture.

273. Enriched Media

a. BLOOD AGAR. Add 5 to 10 ml of sterile defibrinated whole blood to 100 ml of blood agar base, infusion agar or nutrient agar which has been melted and cooled to 50° C. Mix well, pour plates or prepare slants, and incubate to test sterility. The base medium must contain 0.5 percent sodium chloride to prevent hemolysis of the blood. The medium given in paragraph 272j contains this amount. Human, rabbit, sheep, or horse blood may be used. When attempting to isolate hemolytic streptococci from the respiratory passages, however, sheep blood is preferred, because it contains the rabbit serum (RS) factor which promotes growth of these organisms and, unlike human, rabbit, and horse blood, sheep blood does not support growth of the confusing *Hemophilus hemolyticus* which is frequently present in the respiratory tract. Horse blood does not contain the RS factor.

b. SERUM AGAR. Add 10 ml of sterile normal serum (rabbit, sheep, horse, or pooled human) to 100 ml of blood agar base or infusion agar that has been melted and cooled to 50° C. Distribute

into sterile tubes and slant, or pour into plates. Incubate to test sterility. Sterile ascitic or hydrocele fluid may be used instead of serum.

c. **ENRICHED LIQUID MEDIA.** Liquid media may also be enriched with blood or serum as above. It must be remembered that serum and ascitic fluid contain maltase, which must be inactivated by heating at 60° C. for 1 hour if these fluids are to be added to media containing maltose; otherwise a falsely positive fermentation of maltose will be obtained.

d. **CHOCOLATE BLOOD AGAR.**

- (1) Add 5 ml of sterile defibrinated blood (any species) to 100 ml of melted blood agar base (par. 272j) cooled to 50° to 55° C. Mix carefully to avoid bubbles and slowly raise the temperature to 85° or 90° C. Pour into plates or tube, and slant. Incubate to test sterility. In appearance this medium is chocolate brown.
- (2) An excellent chocolate agar may be prepared with dehydrated hemoglobin. Double strength hemoglobin is prepared by dissolving 2 grams of dehydrated hemoglobin in 100 ml of distilled water. Place the hemoglobin in a dry flask, add the water while the flask is being agitated vigorously. Shake the suspension intermittently for 10 to 15 minutes to break up all aggregates and effect complete solution. Filter through cheesecloth and autoclave for 15 minutes at 15 pounds pressure (121° C.). The sterile solution may be stored. In preparing the chocolate agar, use **DOUBLE** strength blood agar base (par. 272j), i.e., use twice as much of the base, per volume of water, as is ordinarily used in preparing blood agar. Adjust the temperatures of the hemoglobin solution and the double strength agar to 50° to 60° C. before mixing. Mix in equal volumes and shake without forming air bubbles to insure suspension of the hemoglobin before pouring plates.

Caution: The pouring of enriched agar into sterile petri dishes must be done with the utmost caution. Since these plates are incubated to test their sterility, the presence of a single microscopic colony on the surface will result in gross contamination if, in streaking the plate, the inoculating loop is drawn through such a colony.

274. Biochemical Media

a. **BROTH BASE (FOR FERMENTATION TESTS).**

(1)

Pancreatic digest of casein-----	1.0 gm
Sodium chloride -----	0.5 gm

- (2) Dissolve in 100 ml of distilled water, add 1 gm of the appropriate carbohydrate (0.5 gm in the case of salicin) and adjust the reaction to pH 7.4. Add 1 ml of a 1.6 percent alcoholic solution of bromcresol purple or other indicator. Distribute into fermentation tubes. Fermentation vials need only be added to dextrose tubes since determination of gas production is necessary only for dextrose. Autoclave for not more than 15 minutes at 15 pounds. Cool quickly.
- (3) The dehydrated base, with phenol red indicator, is available as a standard item.
- (4) For the rarer sugars and for all the carbohydrates in laboratories performing infrequent tests, the following is recommended:
 - (a) Sterilize the base in screw-capped tubes or bottles by autoclaving. Tighten the caps after cooling, and store at room temperature.
 - (b) Sterilize 10 percent solutions of the sugars (5 percent in the case of salicin) in screw-capped vials by autoclaving (10 minutes at 12 pounds) or preferably by filtration. These can be stored at room temperature and a working set of the various sugars kept handy in a rack or block.
 - (c) For use, pipette 5 ml amounts of the sterile base aseptically into sterile plugged Wassermann tubes and add 0.5 ml amounts of the appropriate sterile sugar solutions. It is recommended that enough tubes be made in advance to permit overnight incubation prior to use, thus forestalling the use of contaminated media.
- (5) Unless fair growth of the organism occurs in the medium, the broth should be enriched by the aseptic addition of a few drops of sterile serum or ascitic fluid to each tube. For maltose fermentation, the serum or ascitic fluid should be inactivated by heating at 60° C. for 1 hour. (See par. 273c.) Note, also, the growth stimulating influence of added 0.2 to 0.4 percent agar for anaerobes, gonococci, meningococci, and certain other organisms (see medium presented in par. 271e).

b. SEMISOLID FERMENTATION-MEDIUM BASE. A medium such as that described immediately above but to which 0.2 percent agar has been added is superior to the broth base for some of the more fastidious organisms, such as gonococci and meningococci, partly because the various levels of the semisolid medium provide different degrees of oxygen tension. If small amounts of acid produc-

tion are significant, phenol red or brom thymol blue are better indicators than bromcresol purple. (See par. 271e.)

c. **CLARK AND LUBS MEDIUM (FOR MR-VP TESTS).** The following medium is prepared for the methyl red and Voges-Proskauer tests:

Pancreatic digest of casein or peptone-----	0.7 gm
Dextrose -----	0.5 gm
Dipotassium phosphate (K_2HPO_4) -----	0.5 gm
Distilled water -----	100.0 ml

Dissolve by heating, tube in 10 ml amounts, and sterilize in the autoclave at not over 15 pounds pressure. The pH need not be adjusted.

d. **RUSSELL'S DOUBLE SUGAR AGAR (FOR ENTERIC ORGANISMS).** Russell's double sugar agar contains 1 percent lactose, 0.1 percent dextrose, nutrient agar, and phenol red indicator. The agar is slanted, but it is important that the tubes also possess a deep butt. Growth from an isolated colony is stabbed into the medium and streaked on the surface. Organisms fermenting lactose produce sufficient acid to change the reaction of the entire tube of medium. Those fermenting only the small amount of dextrose present do not produce enough acid to neutralize the alkaline end products at the surface of the medium, although acid is indicated in the butt. Gas is caught in the depths of the medium. Since the media given in *e* contain added chemicals for indicating production of hydrogen sulfide and, in *e* (2) below an additional sugar, these modifications of Russell's medium are preferred.

e. **KLIGLER'S IRON AGAR AND TRIPLE SUGAR IRON AGAR.**

- (1) *Kligler's iron agar (KIA).* This medium contains the same ingredients as Russell's double sugar agar, and fermentation of lactose and dextrose is indicated in the same manner. It also contains compounds which enable one to determine the ability of an organism to produce hydrogen sulfide, indicated by blackening in the medium. This is due to the reduction of sulfur-containing compounds (peptones and sodium thiosulfate) to form hydrogen sulfide which combines with iron salts to form iron sulfide. KIA is available in dehydrated form. The freshly prepared medium gives the best results but screw-capped tubes delay drying and increase the period of utility. (See table XXX.)
- (2) *Triple sugar iron agar.* This medium is prepared by adding 1 gm of sucrose to each 100 ml of melted Kligler's iron agar. Acidity throughout the medium indicates fermentation of either lactose or sucrose. See footnote to table XXX.

f. **NUTRIENT GELATIN.** Add 12 gm of granular gelatin to 100 ml of cold distilled water. Allow to stand for 15 to 30 minutes. Then heat carefully until the gelatin is dissolved. Add the appropriate weight (for 100 ml) of the dry ingredients of nutrient broth (par. 271a), special broth (par 272i) or infusion broth (par. 271c). Adjust the reaction to pH 6.8 to 7.0. Tube, autoclave and cool quickly. Iron gelatin is prepared by adding a strip of iron (not galvanized) or an acid-cleaned nail to a deep (7 to 8 cm) tube of nutrient gelatin before autoclaving. When this medium is used for detecting gelatin liquefaction by anaerobes, it is not necessary to incubate in an anaerobic jar after inoculation.

g. **LITMUS MILK.** This is available in dehydrated form but may be prepared as follows: Add 100 ml of fresh skimmed milk of good quality to 0.5 gm of litmus. Ten gm of dehydrated skimmed milk and 100 ml of water may be used. Tube and autoclave. For the "stormy fermentation" test for *Clostridium perfringens* (Welchii), add 0.1 gm of pancreatic digest of casein or peptone.

h. **INDOL TEST MEDIUM.**

Pancreatic digest of casein or tryptone-----	2.0 gm
Sodium chloride -----	0.5 gm
Distilled water -----	100.0 ml

Dissolve by moderate heating, tube, and autoclave. Adjustment of the pH is not necessary.

i. **LEAD ACETATE AGAR (FOR HYDROGEN SULFIDE PRODUCTION).**

Tryptone (Bacto) -----	20 gm
Agar -----	15 gm
Distilled water -----	1,000 ml
Dextrose (25 percent aqueous solution)-----	4 ml
Lead acetate (0.5 percent aqueous solution)-----	100 ml

Dissolve the tryptone and the agar in the water, and adjust the reaction to pH 6.7 to 7.0. Add the dextrose solution and the lead acetate solution, tube, and autoclave. The medium is not slanted after sterilization.

This medium may be made as a semisolid agar. It may also be prepared without the addition of lead acetate and used with lead acetate test strips as described in paragraph 285b. Lead acetate inhibits the growth of some organisms.

j. **NITRATE BROTH (FOR NITRATE REDUCTION TEST).**

Pancreatic digest of casein or peptone-----	1.0 gm
Potassium nitrate (nitrite free) -----	0.1 gm
Distilled water (ammonia-free) -----	100.0 ml

Dissolve by heating, adjust the pH to 7.0, tube, and autoclave. One-tenth gram of potassium nitrate added to 100 ml of nutrient broth is also satisfactory.

k. CITRATE AGAR (SIMMONS).

Magnesium sulfate	0.2 gm
Sodium chloride	5 gm
Ammonium acid phosphate (NH ₄ H ₂ PO ₄)	1 gm
Sodium citrate (5½ H ₂ O)	2.77 gm
Distilled water	1,000 ml
Agar	20 gm
Brom thymol blue (1.5 percent alcoholic solution) ...	10 ml

Dissolve the chemicals in the distilled water; add the agar and heat to dissolve. Adjust the reaction to pH 7.2, and add the brom thymol blue solution. Tube, autoclave, and slant.

This medium is available in dehydrated form. It tests the ability of an organism to grow in the absence of any assimilable organic carbon compound. Thus *E. coli* and members of the *Shigella* group fail to grow in it, whereas *A. aerogenes* and many of the *Salmonellae* can utilize the sodium citrate for carbon and will grow. Growth, with consequent production of alkaline end products, is evident by a bluing of the medium often before actual colonies are observed. If other than the dehydrated medium is prepared, it should be tested with an organism which grows on the medium as well as one which does not grow.

l. TARTRATE AGAR (JORDAN).

Agar	20 gm
Peptone	10 gm
Sodium potassium tartrate	10 gm
Sodium chloride	5 gm
Distilled water	1,000 ml
Phenol red (0.2 percent alcoholic solution)	12 ml

Dissolve all ingredients but the dye by heating, and adjust the reaction to pH 7.4. Add the phenol red solution, tube, and autoclave.

The medium should be tested with known cultures of *S. typhimurium*, *S. enteritidis*, *S. paratyphi*, and *S. schottmuelleri* before using routinely.

m. SODIUM HIPPURATE BROTH (FOR HYDROLYSIS TEST WITH BETA-HEMOLYTIC STREPTOCOCCI). To special broth (par. 272i) or infusion broth add exactly 1.0 percent of sodium hippurate. Tube the medium, and mark the level of the medium in each tube with a heat-resistant wax pencil or in some other manner, and autoclave. After the culture has grown, immediately before testing for hydrolysis with ferric chloride reagent (par. 293), make the medium up to its original volume by the addition of distilled water.

n. POTATO SLANTS. Select large white potatoes; peel and scrub them thoroughly under running water. Cut cylinders from the potato with a cork borer. Cut these obliquely into wedge-shaped pieces, and place them in running water overnight to reduce

acidity and to prevent darkening. Place in tubes, and to each add about 2 ml of distilled water; autoclave.

o. UREASE TEST MEDIA.

(1) For rapid screening test.

Urea	2.0 gm
Potassium acid phosphate (KH_2PO_4)	0.1 gm
Dibasic potassium or sodium phosphate (K_2HPO_4 or Na_2HPO_4)	0.1 gm
Sodium chloride	0.5 gm
Ethyl alcohol (95 percent)	1.0 ml
Distilled water	99.0 ml

The reagent is adjusted to pH 7.0 and 0.5 ml of a 0.2 percent aqueous solution of phenol red added. Sterilization is unnecessary, but if done it should be by filtration. The unsterilized medium may be stored (refrigerated) for several weeks. Discard if it turns pink. (Prepare phenol red solution as in par. 271e(3).)

- (2) For 24-hour test.** The medium used for this test is much more highly buffered. It is available in dehydrated form as a standard item. Prepare according to directions given on the bottle. It should be sterilized by filtration only.

275. Special Media

a. DEXTROSE (GLUCOSE) BROTH. To 1,000 ml of extract or infusion broth add 10 gm of dextrose and heat slightly until dissolved. If necessary, adjust the reaction to the original pH of the broth. Sterilize in the autoclave.

b. DEXTROSE (GLUCOSE) AGAR. Dissolve 1 percent of dextrose in melted extract or infusion agar. Adjust the reaction if necessary. Sterilize in the autoclave.

c. GLYCEROL AGAR (FOR TUBERCLE BACILLI). To 1,000 ml of melted infusion agar add 30 ml of pure glycerol. Mix thoroughly, and adjust the reaction to pH 7.2. Tube, autoclave, and slant.

d. STANDARD EXTRACT BROTH (FOR WATER ANALYSIS).

Peptone (Bacto)	5 gm
Beef extract (Bacto)	3 gm
Distilled water	1,000 ml

Dissolve the peptone and beef extract in the water. If necessary, adjust the reaction to pH 6.4 to 7.0. Autoclave.

e. STANDARD EXTRACT AGAR (FOR WATER ANALYSIS) (see also *i* below).

Standard extract broth (as above)	1,000 ml
Agar	15 gm

Add the agar to the broth and dissolve. Mix thoroughly, and if necessary, adjust the reaction to pH 6.4 to 7.0. Distribute, and sterilize in the autoclave.

f. **STANDARD LACTOSE BROTH (FOR WATER ANALYSIS).** To standard extract broth (as above) add 0.5 percent of lactose. Adjust the reaction to pH 6.4 to 7.0 (preferably pH 6.9). Tube, and autoclave. This medium is available in dehydrated form.

g. **EOSIN METHYLENE BLUE (EMB) AGAR (LEVINE) (FOR WATER ANALYSIS).**

Peptone	10 gm
Potassium phosphate dibasic	2 gm
Agar	15 gm
Distilled water	1,000 ml

Mix ingredients and dissolve by boiling. Restore the volume with distilled water. No adjustment of reaction is necessary. Distribute 100-ml amounts into flasks and autoclave for 15 minutes at 15 pounds. Just prior to use, melt the above, and to each 100 ml add aseptically the following mixture:

Lactose (20 percent sterile aqueous solution)	5 ml
Eosin, yellowish (2 percent aqueous solution)	2 ml
Methylene blue (0.325 percent aqueous solution)	2 ml

These quantities of eosin Y and methylene blue are based on the use of certified dyes with dye content of 85 and 90 percent, respectively. Mix thoroughly and pour into plates. Allow the medium to harden and incubate the plates to test their sterility.

It is permissible to add all the ingredients to the stock agar at the time of preparation, distribute the medium into flasks, and sterilize in the autoclave. From this medium plates may be poured as needed. This medium is available in dehydrated form.

h. **BRILLIANT GREEN LACTOSE BILE BROTH (FOR WATER ANALYSIS).**

Peptone (Bacto)	10 gm
Lactose	10 gm
Distilled water	500 ml

Dissolve, and add —

Fresh beef bile	200 ml
(or 20 gm of dehydrated beef bile dissolved in 200 ml of distilled water).	

Then add —

Distilled water q. s. ad	975 ml
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Adjust to pH 7.4, and add —

Brilliant green (0.1 percent aqueous solution)	13.3 ml
Distilled water, q. s. ad	1,000 ml

Filter through cotton, tube, and autoclave. The final reaction should be not less than pH 7.1 and not more than pH 7.4. This medium is available in dehydrated form.

i. **TRYPTONE DEXTROSE (GLUCOSE) EXTRACT MILK AGAR.**

(1) *For milk analysis:*

Agar	15 gm
Beef extract	3 gm
Tryptone (Bacto)	5 gm

Dextrose (glucose)	1 gm
Distilled water	1,000 ml

Dissolve by boiling. Restore the volume by adding distilled water. Adjust the reaction to pH 7.0. Add —

Skimmed milk	10 ml
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Dispense measured amounts — 100 or 200 ml in flasks or 10 to 12 ml in test tubes. Autoclave.

- (2) *For water analysis.* Prepare as above but omit the addition of skimmed milk. This medium is available in dehydrated form.

j. FLETCHER'S MEDIUM (FOR LEPTOSPIRA). A 12 percent solution of sterile rabbit serum in sterile distilled water is heated to 50° C. Aseptically add 6 ml of 2.5 percent sterile, melted nutrient agar (pH 7.4), or 7.5 ml of 2 percent nutrient agar, to every 100 ml of the serum water. Tube in 5-ml quantities, and sterilize by heating at 56° C. for 1 hour on 2 successive days. Incubate for sterility.

k. EGG YOLK AGAR (FOR CLOSTRIDIA).

- (1) Scrub the shell of a fresh hen's egg. Place the egg in a 1:1000 aqueous solution of mercuric chloride (HgCl_2). Put on sterile rubber gloves. Wash the egg with alcohol, then break and discard the white. Aseptically aspirate the yolk with a sterile syringe and needle. Add the yolk to an equal volume of a sterile physiological saline solution and mix well. Before use, test sterility by plating.

- (2) *Prepare agar medium as follows:*

Pancreatic digest of casein	4.0 gm
Dipotassium phosphate (K_2HPO_4)	0.5 gm
Sodium chloride	0.2 gm
Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.01 gm
Dextrose	0.2 gm
Powdered agar	2.5 gm
Distilled water	100.0 ml

Dissolve and melt, adjust pH to 7.6 and autoclave. When cooled to 50° C., add 10 ml of yolk suspension (1) to each 90 ml of agar medium, mix and pour plates.

- (3) Organisms which produce lecithinase (certain clostridia) show an opacity around the colony. Some workers use pooled human serum (20 ml to 80 ml of agar medium) in place of the egg yolk suspension.

276. Miscellaneous Solutions

a. PHYSIOLOGICAL SALT SOLUTION (NORMAL SALINE SOLUTION).

Sodium chloride	8.5 gm
Distilled water	1,000 ml

b. **BUFFER SOLUTION, pH7.4±.** (Refer to par. 122 for buffer solutions of other pH values.)

Sodium dihydrogen phosphate (NaH_2PO_4)	28.81 gm
Disodium hydrogen phosphate (Na_2HPO_4)	125 gm
Distilled water q. s. ad.	1,000 ml

c. **SODIUM CHLORIDE SOLUTION (BUFFERED).**

Buffer solution (above)	20 ml
Sodium chloride	8.5 gm
Distilled water q. s. ad.	1,000 ml

d. **SODIUM CITRATE — SODIUM CHLORIDE SOLUTIONS (ANTI-COAGULANT).** To physiological salt solution (above) add 1, 2, or 10 percent sodium citrate. Sterilize in the autoclave.

When used as an anticoagulant of blood, a final concentration of not less than 0.25 percent sodium citrate is required; therefore, to each 10 ml of blood add 3.3 ml of the 1 percent solution, 1.4 ml of the 2 percent solution, or 0.26 ml of the 10 percent solution.

e. **POTASSIUM OXALATE SOLUTION (ANTICOAGULANT).**

Potassium oxalate	2 gm
Sodium chloride	6 gm
Distilled water	100 ml

To prevent coagulation add 1 ml of this solution to 10 ml of blood. A convenient method of collecting oxalated blood, as for Tillett and Garner's fibrinolytic test, is to place 1-ml amounts of a 2 percent solution of potassium oxalate (sodium chloride not required) in small, wide-mouth bottles, which are plugged with cotton and sterilized in the hot-air oven. The oxalate solution evaporates to dryness. Ten milliliters of freshly drawn blood is placed in the bottle, and immediately rotated to dissolve the oxalate. By this method dilution of the blood is avoided.

Caution: Since potassium oxalate is somewhat bactericidal, use sodium citrate as an anticoagulant for specimens that require cultural studies.

f. **SODIUM CARBONATE SOLUTION.** The addition of about 2 gm of sodium carbonate (washing soda) per liter of water serves to prevent corrosion of instruments during boiling.

g. **DISINFECTANT SOLUTION (FOR DESK JAR USE).** For use in jars and cylinders into which contaminated pipettes are placed, use a 2 or 5 percent solution of liquor cresolis compound *U.S.P.* in tap water.

Section V. BIOCHEMICAL TESTS

277. Carbohydrate Fermentations

Fermentation tests play an important part in the identification of bacteria. Fermentation media are usually prepared by adding 0.5 percent of the carbohydrate (1 percent in the case of lactose)

to a sugar-free basic medium such as tryptose broth. To detect the production of acid or alkali, an acid-base indicator, such as phenol red or brom cresol purple, is usually added. To detect gas formation, a small inverted vial is placed in each tube of medium.

The more commonly used differential carbohydrates are dextrose (glucose), maltose, sucrose (saccharose), lactose, xylose, arabinose, rhamnose, mannitol (mannite), dulcitol (dulcitol), sorbitol (sorbite), inositol (inosite), glycerol (glycerin), glycogen, dextrin, starch, inulin, and salicin.

278. Methyl Red (MR) Test

The test medium (Clark and Lubs medium) contains dextrose and phosphate buffer. The test is performed after incubation of the culture for 3 to 4 days.

Add about 5 drops of indicator (0.04 percent solution of methyl red in 60 percent alcohol). Read immediately. A red color (acid) is a *positive* test; a yellow color (alkaline) is a *negative* test; an orange color is an *intermediate* test. This test measures quantitatively the ability of an organism to produce acid from dextrose. If sufficient quantities of acids accumulate, the buffer capacity of the medium is overcome more or less completely and a *positive* or an *intermediate* test is obtained.

279. Voges-Proskauer (VP) Test

a. A culture in Clark and Lubs medium, incubated for 24 to 48 hours at 37° C. is used. Add to the culture an equal volume of 10 percent sodium or potassium hydroxide solution. Replug the tube and shake vigorously at intervals for ½ hour. A *positive* test is indicated by a pink or red color. If negative, incubate at 35° C. for 18 to 24 hours, and reread.

b. Another method that gives more rapid and more reliable results is that of Barrett. This test is performed by adding 0.6 ml of 5 percent alpha naphthol in absolute ethyl alcohol and 0.2 ml of a 40 percent sodium or potassium hydroxide solution to 1 ml of culture after incubation for 24 to 48 hours. The color reaction is as above.

c. By either method, a positive test is an indication of the accumulation of acetyl-methyl-carbinol as an end product of dextrose fermentation. It is sometimes referred to as the acetyl-methyl-carbinol (AMC) test. (See table XXXI.)

280. Urease Tests

a. RAPID UREASE TEST. This is a simple and rapid screen test which can be performed on all gram-negative rods showing pos-

sible *Salmonella* or *Shigella* reactions in double or triple sugar agar tubes. Inoculate heavily 0.5 ml amounts of the medium given in paragraph 274o(1) and place the tubes (including an uninoculated control) in a water bath at 35° to 36° C. *Proteus* organisms hydrolyze the urea rapidly and release ammonia which is indicated by reddening of the indicator, usually within 15 minutes to 2 hours. The more highly buffered medium discussed in (b) below also may be used for a rapid screen test, but tubes showing a negative reaction should be incubated overnight before discarding as negative.

b. TWENTY-FOUR HOUR UREASE TEST. The medium given in paragraph 274o(2) is recommended for this test. Possible *Salmonella* organisms showing no reaction in the rapid urease test are inoculated into this medium at the same time other tubes for biochemical tests are inoculated. In addition to species of *Proteus*, some paracolon organisms also hydrolyze urea, but usually do so more slowly than the *Proteus*.

281. Kligler's Iron (And Other Combined Sugars) Agar Reactions

The reactions indicated by these media are given in paragraphs 274d and e. Tabulated reactions of enteric organisms are given in table XXX.

282. Litmus Milk Reactions

a. Litmus milk has a differential value based on lactose fermentation, reduction of litmus, coagulation of milk protein, and digestion of the casein. Different degrees of acidity (lactose fermentation) or alkalinity are indicated by the color of the indicator. Reduction of litmus is indicated by its decolorization. Coagulation is detected by noting clotting of the milk (curd formation) and may be due to the formation of acid, producing a firm clot that does not shrink, or to the action of a rennet-like enzyme, producing a soft contracting clot unaccompanied by marked acidity. Digestion of the casein, indicated by a partial or complete clearing of the milk, may or may not be preceded by coagulation. Brom cresol purple milk has the same uses, but the indicator does not indicate reduction as does litmus.

b. "Stormy fermentation" is the term used to describe a reaction in milk such as that produced by *Clostridium perfringens* (*Welchii*). It is the result of rapid coagulation followed by active gas production, which results in tearing up of the clot. This reaction is not as specific for *Cl. perfringens* as formerly supposed, and some strains of *Cl. perfringens* produce this effect slowly and only under special conditions.

283. Indol Test

a. REAGENTS.

- (1) Prepare oxalic acid test paper by soaking filter paper in a warm saturated aqueous solution of oxalic acid. Allow the paper to air dry, then cut into small strips. Store in tight containers.

- (2) Kovac's reagent is prepared as follows:

Paradimethylaminobenzaldehyde	5 gm
Amyl alcohol	75 ml
Hydrochloric acid	25 ml

Heat the alcohol and the aldehyde in a water bath at 50° to 60° C. until dissolved. Cool and add the acid slowly. Store in a glass-stoppered brown bottle in the dark.

b. TEST.

- (1) Inoculate a tube of indol test medium (see par. 274*h*).
- (2) Insert a strip of oxalic acid test paper (*a*(1)) into the tube with the upper portion held in place by the stopper. Do not allow paper to contact the medium. This is the Gnezda's test. During the growth of the culture, a light pink color on the paper indicates indol production.
- (3) After the paper is observed for 48 to 72 hours, if still negative, the medium should be tested with Kovac's reagent (*a*(2)) above. Add 0.5 to 1.0 ml of the reagent and shake gently. Allow the reagent to rise to the surface. A dark red color in the reagent is a positive test; no change in color, a negative test.
- (4) The indol tests depend upon the liberation of indol from tryptophane by organisms such as *E. coli*, but not by others such as *A. aerogenes* and *Salmonella* species.

284. Cholera Red (Nitroso-indol) Test

Add a few drops of concentrated sulfuric acid to a 24- to 48-hour peptone water culture. As in the indol test, the culture medium must contain tryptophane. Moreover, the peptone-water medium used for cholera red tests must contain an adequate amount of nitrate, but not *an excess*. A tryptone suitable for the test may be selected by testing the available lots with known cholera red positive strains. To insure a medium that will be satisfactory for the test any peptone that gives good indol tests may be used with the addition of 0.001 percent (not more or less) sodium nitrate. The appearance of a red color is a *positive* test, indicating the presence of indol and nitrite. This test is not strictly specific for the cholera vibrio.

285. Hydrogen Sulfide Production Tests

a. Kligler's iron agar or triple sugar iron agar indicate hydrogen sulfide production by blackening (see par. 274e).

b. In conjunction with the indol test (see par. 283), a paper strip impregnated with lead acetate also may be similarly suspended over the culture in the same tube. These are made by impregnating similar strips of filter paper in a warm, saturated solution of neutral lead acetate. They also may be dried and stored. Do not allow paper to contact the medium. During incubation of the culture, observe daily for browning or blackening of the paper. This indicates that hydrogen sulfide has been liberated from the peptones in the medium.

286. Gelatin Liquefaction

a. Inoculate nutrient gelatin by repeated stabbing with a straight needle bearing the culture being studied. Incubate at 35° C., together with a tube of the uninoculated medium. Examine daily for several days to a week or more for the presence of liquefaction, indicating the production of the proteolytic enzyme gelatinase. Test by holding the incubated inoculated and uninoculated tubes together in an ice bath. Observe the inoculated tube for fluidity as soon as the uninoculated tube gels. Reincubate both tubes if liquefaction is not observed.

b. Inoculated tubes may be incubated at 20° to 22° C., and examined directly for liquefaction, but the reaction is generally delayed in comparison with those tubes incubated at higher temperatures.

287. Citrate Utilization

The surface of Simmons' citrate agar (par. 274k) should be inoculated lightly, together with a tube of nutrient agar similarly inoculated. Incubate each and examine daily. Evidence of growth is a positive test and generally is accompanied by a bluing of the medium (see par. 274k). If no growth occurs, examine the nutrient control tube to check viability of the culture. The citrate agar is inoculated lightly to minimize the effect of accompanying nutrients.

288. Tartrate Utilization

Stab inoculate Jordan's tartrate agar. Incubate for several days, and examine daily. An acid reaction at the bottom of the tube is a positive reaction.

289. Nitrate-reduction Test

a. REAGENTS.

(1) Solution A.

Sulfanilic acid (ACS)	2 gm
Acetic acid (30 percent)	250 ml

(2) Solution B.

Alpha naphthylamine	1 gm
Acetic acid (30 percent)	200 ml

Filter through absorbent cotton.

b. PROCEDURE. Culture the organism in nitrate broth for 1 to 5 days, checking daily. Add about 0.5 ml of solution A, then 0.5 ml of solution B. Mix and examine.

c. INTERPRETATION. A *positive* reaction for nitrite is indicated by a red, purple, or maroon color; *negative*, no color change.

Caution: In certain cultures all the nitrate and nitrite may be consumed, resulting in a negative test for nitrite. Therefore, if no color is obtained, test for nitrate by adding a pinch of zinc dust to the culture containing the nitrite reagents and allow the tube to stand a few minutes. If nitrate is present, it will be reduced to nitrite and show the characteristic color reaction.

290. Methylene Blue Reduction Test

(A special test by same name is used for rapid grading of milk.)

To a 24-hour broth culture add a sufficient amount of a 1 percent aqueous solution of methylene blue to make 1 part of reagent to 50 parts of culture. Incubate at 37° C., observing at hourly intervals for evidence of reduction (decolorization).

291. Oxidase Test (for Neisseria)

This test is extensively used in selecting colonies for isolation and identification in the cultural diagnosis of gonococcus and meningococcus infections.

a. REAGENT. Use an approximately 1 percent aqueous solution (freshly prepared) of para-amino-dimethyl-aniline-monohydrochloride (dimethyl-paraphenylene-diamine-hydrochloride). The tetramethyl compound also may be used; it is somewhat less toxic to the bacteria but much more expensive.

b. PROCEDURE. Flood the surface of the plate culture with 0.5 to 1.0 ml of the reagent. Colonies of oxidase positive organisms first become pink, then gradually darker through purple to black. The colony itself should show this color. Discoloration of the surrounding medium is not significant. Isolations from colonies must

be made while they are still pink; later the organisms may no longer be viable.

c. **INTERPRETATION.** The reaction is not strictly specific. Non-pathogenic *Neisseria* and some other bacteria are oxidase positive.

292. Bile-solubility Tests (for Pneumococci)

a. **BILE SOLUBILITY.** Autoclave fresh undiluted beef bile, filter through paper, and reautoclave. To 1.0 ml of the turbid broth culture add 0.2 ml bile. Use an untested portion of the culture for a turbidity control. Observe for clearing of the culture over a period of 1 hour at room temperature.

b. **DESOXYCHOLATE SOLUBILITY.** Prepare a 10 percent aqueous solution of sodium desoxycholate (a bile salt), adding 1:50,000 Merthiolate as a preservative. Add 2 drops of the reagent to 1 ml of broth culture (not dextrose broth). Observe and read as above.

c. **SLIDE TECHNIC (FOR CULTURES THAT CANNOT BE SATISFACTORILY TESTED BY ABOVE METHODS BECAUSE OF CONTAMINATION, ACIDITY, OR OTHER REASONS).** On a slide place 1 loopful each of reagent, methylene blue stain, and broth culture (or suspension from colony). Observe microscopically immediately and at intervals for a period of $\frac{1}{2}$ to 1 hour, if necessary, for disappearance of the pneumococci.

d. **PLATE TECHNIC.** Dust powdered sodium desoxycholate over some of the suspicious colonies on a blood-agar plate. If the colonies are composed of pneumococci, they will "disappear" (lyse) after 10 to 15 minutes.

Caution: The bile-solubility test is not always satisfactory. Negative or positive "Quelling" reactions with pneumococcus grouping sera often are more satisfactory in attempting to distinguish between pneumococci and alpha streptococci. (See par. 304.)

293. Hippurate-hydrolysis Test (Ayers and Rupp) (for Beta-hemolytic Streptococci)

Dissolve 12 gm of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in 10 ml of a 2 percent aqueous solution of hydrochloric acid. Transfer 0.8 ml of culture in sodium hippurate broth to a small test tube (Wasserman tube) and add 0.2 ml of the reagent. Mix immediately and observe after 10 to 15 minutes. A permanent precipitate indicates the presence of benzoic acid (hydrolysis of hippurate).

Caution: Since sodium hippurate is first precipitated and later redissolved by the amount of reagent specified, and since benzoic acid is also redissolved by a greater excess of the reagent, it is necessary to have the reagent and medium balanced and to meas-

ure the amounts used in the test quite accurately. If the medium has evaporated somewhat during storage and incubation, the volume should be restored by addition of distilled water at the time of making the test. (See par. 274 *m.*) A control test of the sterile medium should always be made. If the culture is quite turbid, to confuse the reading of the result, it should be centrifuged and the clear supernatant used for the test.

294. Streptokinase (Fibrinolytic) Test for Beta-Hemolytic Streptococci

Dilute 0.2 ml of oxalated human plasma (add 0.02 gm of potassium oxalate to 10 ml of blood and centrifuge) with 0.8 ml of physiological saline solution. Add 0.5 ml of a young (18- to 24-hour) turbid, broth culture of the streptococcus to be tested. Mix immediately, and add 0.25 ml of a 0.25 percent aqueous solution of calcium chloride. Mix and place in a water bath at 37° C. In about 10 minutes there should be a solid coagulum. Observe frequently and note the time when the contents of the tube become completely fluid.

Caution: The broth should not contain more than 0.1 percent of dextrose but may be enriched by the addition of a few drops of serum or ascitic fluid if necessary. The human plasma must be of known sensitivity. Plasma from certain persons, notably those who may have recovered recently from hemolytic streptococcal infections, is not suitable for the test.

295. Coagulase Test (for Staphylococci)

To 0.5 ml of heparinized human plasma, add 0.5 ml of a 24-hour culture of staphylococci in broth or a large loopful of growth from an agar slant. Incubate in a water bath at 37° C. and observe frequently. If coagulation of the plasma occurs within 3 hours, the test may be regarded as positive; the shorter the period required, the more strongly positive is the result.

296. Catalase Test

Place a drop of concentrated hydrogen peroxide (25 to 30 percent) on a clean glass slide. Emulsify a colony or a small amount of growth in the drop and observe for bubbling. Members of the tribe *Streptococceae* do not produce catalase whereas micrococci, staphylococci, and diptheroids do produce this enzyme which liberates oxygen from the peroxide and causes bubbling. Use nichrome wire since platinum sometimes causes bubbling. If the colony is taken from blood agar, be careful not to include any of the medium in the drop since blood causes liberation of oxygen.

The reagent should be stored in the refrigerator when not in use.
(See par. 336.)

Section VI. SEROLOGICAL METHODS

297. Preparation of Antigens

The reliability of a serological test is dependent on having a satisfactory antigen. For agglutination reactions such antigens are dependent not only on the specificity of the strain used but also on the dissociative phase in which the organism exists. Although some strains may be better antigens than others, in general, care should be exercised to use a smooth, motile (or nonmotile) strain that is typical in all respects. Antigens used for agglutination are suspensions of either living or killed organisms.

a. LIVING CULTURES AS ANTIGENS. Very satisfactory antigens can be prepared by growing an organism in broth and using it without alteration. If the broth culture is centrifuged and the organisms are resuspended in physiological (normal) saline solution, the possible detrimental effects of constituents of the medium, or of complications in reading due to color of the broth, are eliminated.

If the culture is grown on a suitable solid medium, such as an agar slant or plate, the organisms may be washed off with saline solutions and used without further treatment. If soluble portions of the medium interfere with the test the suspension may be centrifuged and the organisms resuspended in saline.

Although living antigens are satisfactory, their use is seldom necessary and should be discouraged because of the danger in handling them.

b. PREPARATION OF KILLED ANTIGENS. A simple killed antigen, which may be used without risk of infection, may be prepared by killing broth cultures or a saline suspension with heat or with chemical reagents.

- (1) *Heat-killed antigen.* The bacterial suspension may be heated in a water bath at 70° to 80° C. for 30 to 60 minutes. To prevent drying of bacteria on the sides of the tube above the suspension, it is recommended that a stopper be inserted above the cotton plug, thus keeping the atmosphere in the tube saturated during the heating process.
- (2) *Formalin-killed antigen.* Usually 0.3 percent final concentration of formalin (formaldehyde U.S.P.) added to the suspension is sufficient to kill the bacteria; more may be necessary if a whole broth culture is used since for-

maldehyde reacts with amino acids and peptones and is partially inactivated by them.

- (3) *Phenol-killed antigen*. Add phenol 0.5 percent concentration to the broth culture or bacterial suspension.

c. PREPARATION OF SPECIAL ANTIGENS. Some antigens are best prepared in a manner that enhances their value as diagnostic agents. Motile bacteria, notably species of *Eberthella* and *Salmonella*, yield additional information if the flagellar (H) antigens and the somatic (O) antigens are studied separately. The flagella contain one set of antigens, and the body of the cell contains a different set. Flagellar antigens withstand formalin but are destroyed by heat, phenol, or alcohol. Somatic antigens are not affected by any of these. Hence, it is possible to prepare an antigen that retains the flagellar components by killing an actively motile culture with 0.3 percent formalin; this antigen necessarily will also have somatic factors present. On the other hand, an antigen containing only somatic factors may be prepared either from a nonmotile strain or from a motile strain, subjecting the latter to treatment that will destroy the flagellar components.

- (1) *Preparation of flagellar (H) antigens*. Select a smooth, typical, actively motile strain; if necessary, transfer it daily for several days to enhance its motility. It should then be grown on a suitable moist agar medium or in broth for 18 hours. If on agar, the growth is washed off with a minimum amount of normal saline solution and may, if necessary, be filtered through cotton or coarse filter paper to remove the coarse particles. If it is necessary to remove soluble constituents of the medium it may be centrifuged and resuspended in saline solution. If a broth culture is used, it may be killed without further treatment, or the cells may be packed by centrifugation and resuspended in saline solution. The bacteria may be killed either before or after centrifugation. In either instance 0.3 percent formalin is added to the concentrated suspension, which is then left at room temperature or at 37° C. for 24 hours. Sterility tests should be made, especially if the preparation is to be kept as a stock antigen. Concentrated stock antigens, so prepared, may be kept refrigerated for months. If necessary, as much as 2 percent formalin may be used as a killing agent, but the antigen must be so diluted that the concentration of formalin is reduced to 0.3 percent or less at the time of use. Also, at the time of use, the turbidity of the antigen should correspond to that of tube No. 3 of the McFarland nephelometer scale. (See par. 328b.)

- (2) *Preparation of somatic (O) antigens (Bien's alcohol method)*. A nonmotile strain of the organism should be used, if available. Grow the bacteria for 18 to 24 hours on a suitable solid medium, preferably one that is not moist. Wash off the growth in a small amount of normal saline solution and add an equal volume of absolute ethyl alcohol; mix well and allow it to stand at room temperature overnight. The next day add normal saline solution equivalent to half the volume of the alcohol-antigen mixture, thus reducing the alcohol concentration to 33 percent. As a preservative add 0.5 percent phenol. The concentrated antigen may be kept for months, but for use must be diluted with saline solution until the alcohol concentration is not more than 12 percent and the turbidity corresponds to that of tube No. 3 of the MacFarland nephelometer scale.
- (3) *Preparation of antigens for special organisms*.
- (a) *Brucella antigens*. These are prepared by growing the organisms on a suitable solid medium. They are killed by heat (80° C. for 1 hour) and preserved with 0.3 percent phenol.
 - (b) *Pasteurella tularensis antigen*. This is prepared by growing the organisms on blood dextrose cystine agar, and killing it in suspension with 0.3 percent formalin.
 - (c) *Proteus OX-19, OX-2 and OX-K Antigens (Weil-Felix)*. These antigens are prepared from nonmotile strains by killing with heat (80° C. for 1 hour) and preserving with phenol, or by the method described above for the preparation of somatic (O) antigens.
 - (d) *Available prepared antigens*. Bacterial antigens for tube agglutination tests may be obtained by requisition from the Army Medical Service Graduate School. Antigens for species of *Salmonella* and *Shigella* are of no practical diagnostic value and therefore are not available for routine issue. These infections are nearly always acute and there is not sufficient time for the production of antibodies in the patient. Diagnosis should be made by isolation of the organism. Then, if agglutinations are desired, the patient's serum should be tested against an antigen prepared with his own strain. Similarly, antigens for the coli-aerogenes group of bacteria, for species of *Clostridium* and *Hemophilus*, for meningococci and for some other groups are of no diagnostic value.

298. Preparation of Agglutinating Sera

a.

- (1) Bacteria, when introduced into the body of an animal, bring about the production of antibodies. Among these antibodies are agglutinins, which may be titrated by means of agglutination tests. Since the agglutinins are specific for their respective antigens, antisera specific for various species or groups of organisms may be obtained.
- (2) The strain of bacteria to be used as an antigen for injection into animals should be selected carefully, using a typical, smooth organism having all of the somatic and flagellar antigenic components desired. The bacterial suspension is prepared, killed, and tested for sterility, as described above, but is frequently used in greater concentration than is an antigen used for the titration of agglutinins.
- (3) The rabbit is the laboratory animal generally used for immunization. The antigen is injected into the marginal ear veins in doses determined by the toxicity of the organism. A suggested schedule for nontoxic strains is to give doses of 0.5, 1.0, 1.5, and 2.0 ml at intervals of 5 to 7 days. Six days after the last injection 1 or 2 ml of blood are taken from an ear vein and the agglutinins in the serum are titrated. If the titer is satisfactory, the rabbit is bled from the heart, and the serum separated and preserved by the addition of 50 percent of glycerin, 0.5 percent of phenol, or 1:10,000 Merthiolate. If the titer is unsatisfactory the rabbit is given additional injections and the titer of the serum rechecked.
- (4) Antigens of toxic strains of bacteria require a modified schedule of injections and it may be necessary to start by giving one or more small subcutaneous inoculations to protect the rabbit against later intravenous injections.
- (5) The prepared serum should be tested against homologous and heterologous antigens for titer and for cross reactions, respectively.

b. Almost all agglutinating sera having practical diagnostic value may be obtained by requisition from the Army Medical Service Graduate School. The fact that they are readily obtainable should not, however, result in their being wasted. Sera having high titers should be diluted prior to their use. They should be used only when indicated. The habit of attempting to classify organisms by serological methods alone or by setting up an un-

known organism against a wide variety of sera is wasteful of serum, confusing, and time-consuming.

299. Macroscopic (Tube) Agglutination Test

The detection of agglutinins in the sera of patients is a highly important procedure for diagnosing infection with certain pathogenic bacteria. The macroscopic or tube agglutination test is recommended. Time is required for antibodies to develop in the blood of a patient. Thus, in typhoid fever, demonstrable agglutinins do not appear until 8 or 10 days after symptoms appear. Thereafter the serum antibodies reach a peak and recede during the following convalescence. It is usually a good policy to obtain two samples of serum (taken at least 10 days apart) from each patient for comparison of titers. Preserved standardized suspensions of the following bacterial species are available as antigens and should be used in routine agglutination tests with patients' sera. The antigens are as follows: *S. typhi* "H" and "O," *S. paratyphi* "H" and "O," *S. paratyphi* B "H" and "O," *Proteus* OX-19, OX-2 and OXK (for rickettsial diseases), *Brucella abortus* and *Pasteurella tularensis*.

a. PROCEDURE.

- (1) Set up a series of clean, clear serological tubes in a rack. Seven tubes usually will be sufficient but 10 may be used if desired.
- (2) Using a seven tube series, place 0.5 ml of physiological saline in each tube.
- (3) Place 0.8 ml of saline in a separate tube and add 0.2 ml of the patient's serum. (It is difficult to pipette accurately less than 0.2 ml of serum.) Mix well and transfer 0.5 ml to the first tube of the series.
- (4) Mix the contents of tube No. 1 and transfer 0.5 to tube No. 2. Mix well.
- (5) Transfer 0.5 ml from tube No. 2 to tube No. 3. Continue this procedure until 0.5 ml of the saline-serum mixture has been removed from tube No. 6. Discard this last 0.5 ml. The last tube No. 7 will contain saline and no serum and thus serves as an antigen control. (See note below.)
- (6) To each of the seven tubes, add 0.5 ml of the antigen. Its turbidity should be that of tube No. 3 of the McFarland nephelometer scale, which is equivalent to 900,000,000 bacteria per ml.
- (7) The final serum dilutions will be 1:20 in tube No. 1, 1:40 in tube No. 2, etc., to 1:640 in tube No. 6. After adding antigen, shake the rack containing the tubes.

Table XXV. Summary of Conditions for Detecting Agglutinins by Macroscopic Test

Organisms	Antigen	Lowest dilution of serum	Incubation*		Readings
			Temperature 0° C.	Time (hr)	
<i>Salmonella typhi</i>	Formalinized H antigens.....	1:20	52	18	At end of incubation period.
<i>S. paratyphi</i> A and <i>S. paratyphi</i> B.....	Phenolized O antigens.....	1:20	52	18	At end of incubation period.
<i>Proteus</i> OX—19, OX—K and OX—2.....	Killed (heat or phenol).....	1:20	52	18	At end of incubation period.
<i>Brucella abortus</i>	Killed (heat or phenol).....	1:20	37	18	At end of incubation period.
<i>Pasteurella tularensis</i>	Killed (formalin).....	1:20	52	18	At end of incubation period.

*If equipment does not permit incubation at 52° C., incubate for 24 to 48 hours at 37° C.

- (8) Incubate all the tubes except those of the *Brucella* series in a water bath at 52° C. for 18 hours. Incubate the *Brucella* series in a water bath at 37° C. for the same period. If a 52° C. water bath is not available, incubation at 37° C. for 24 to 48 hours may be substituted. The tests are read immediately after the indicated periods of incubation. It is not necessary to refrigerate the tubes.

b. **READING.** Observe every tube for clearing of the supernatant fluid and note the amount and character of agglutinated particles. Shake the tube gently. "H" agglutinins produce large flakes of the floccular type which are easily broken up, while "O" agglutinins produce granular or small flaking agglutination. Complete agglutination with complete clearing of the supernatant fluid indicates a "4 plus" reaction. Decreased clumping with increased clouding of the supernatant fluid will be observed. These are read as "3 plus," "2 plus," and last visible agglutination as "1 plus." Tubes resembling the antigen saline control are termed "negative."

c. **REPORTING.** The highest dilution of serum showing a "2 plus" reaction is considered to be the titer and is so reported.

Note. Suspensions of live organisms may be employed as antigens, but extreme caution should be exercised in handling them. When several sera are being tested during 1 day, only one antigen-saline control need be used. This will conserve the supply of antigen.

The tube agglutination test also is used, with known antiserum, for detecting antigens when identifying or typing bacteria (see pars. 306 and 359).

300. Widal Test

The Widal test was originally a microscopic method for testing a patient's serum for the presence of typhoid agglutinins; but this name is frequently applied to macroscopic agglutination tests for the serodiagnosis of other febrile diseases. (See par. 299.)

301. Weil-Felix Reaction

a. This is a macroscopic tube agglutination test used in the diagnosis of rickettsial diseases. The antigen employed is a strain of *Proteus*, originally isolated from the urine of a typhus patient. This organism, *Proteus X*, is not the etiologic agent and except for a common antigen has no relation to species of *Rickettsia* that cause these diseases. As an antigen for agglutination only the non-motile (O) variant of *Proteus X*, living or heat killed, is used. The OX19 strain is used for diagnosis of typhus (epidemic and

murine) and Rocky Mountain spotted fever, and the Kingsbury strain, OXK, is used for tsutsugamushi fever. Agglutinins for *Proteus X* appear in the blood stream during the febrile part of the disease, gradually increase in titer during early convalescence, and disappear in late convalescence. An increase in agglutinin titer is of the greatest significance.

b. Agglutination of *Proteus* OX19 alone cannot be used to distinguish the two types of typhus fever from Rocky Mountain spotted fever. A positive agglutination titer of 1:100 is significant, and an increase in titer during the course of the disease is of primary importance. In typhus a titer of 1:1000 is frequent, and even higher titers are obtained. In Rocky Mountain spotted fever a titer of 1:10,000 has been found. *Proteus* OX2, variant, has been suggested to differentiate Rocky Mountain spotted fever from typhus, but its specificity is of questionable value. Confirmed cases of typhus fever have been reported in which the Weil-Felix test was never positive. (See par. 429.)

302. Macroscopic Slide Agglutination Test

This method is widely used for grouping and typing bacteria on the basis of surface antigens. It is also used as a screening test for detecting antibodies in patients' sera but all positive reactions must be checked with the macroscopic tube agglutination test. The technics used in grouping and typing bacteria differ somewhat with the organisms being studied. In general, the procedure is as follows: A drop of bacterial suspension (antigen) is placed on a glass slide within a 20 by 5 mm area. The area is bounded by an oval or oblong marking made with a wax pencil. A drop of known antiserum is placed nearby in the area and the two drops mixed by tilting the slide back and forth. The tilting also increases the opportunities for contact thus facilitating clumping. An area containing only the bacterial suspension (or the suspension and a drop of serum containing no antibodies) should be studied at the same time for spontaneous clumping. The time used in mixing before declaring a reaction negative differs with the antigens, but in no case should it exceed 5 minutes. It is important to use a wax pencil whose marking does not wash off in contact with the reagents.

303. Microscopic Agglutination Test

Microscopic agglutination tests may be made by mixing droplets or loopfuls of serum and antigen on ringed slides or on cover slips over concave slides. Clumping of the cells is observed under the microscope. As commonly used, the technic is crude. Dilutions

are made by mixing a drop or loop of serum with drops or loops of saline. Serum sensitivity, salt sensitivity, and the natural clumping of some organisms make the reading of results difficult. This method is not advocated.

304. Pneumococcus Typing (Neufeld Reaction)

The procedure given below is no longer widely employed since antibiotics have largely replaced specific antiserum in treatment of pneumococcal infection. On the other hand, the use of typing antisera mixed in groups (as indicated) is valuable for rapidly identifying gram-positive diplococci as pneumococci. It is less time-consuming and more precise than the bile solubility test. The Neufeld or *Quellung* reaction is a rapid method for the typing of pneumococci when they are visibly present in materials, such as sputum, taken directly from patients. The time required is less than 30 minutes. The method should be used only after pneumococcuslike organisms in appreciable numbers have been seen in a stained smear. It is less applicable to the typing of cultures or of material from patients receiving sulfonamides.



- ① Type I pneumococcus in sputum mixed with type II antiserum; no swelling of capsule.
- ② Type I pneumococcus in sputum mixed with type I antiserum; swelling of capsule (Neufeld reaction).

Figure 25. *Diplococcus Pneumoniae* — Showing Neufeld reaction.

a. COLLECTION OF SPECIMEN. A small sample of sputum, coughed up by the patient from the deeper air passages and as free as possible of saliva, is collected in a sterile Petri dish or wide-mouthed bottle. It should be typed without undue delay. Preferably the sample should be collected before beginning treatment with sulfonamides, which interfere with this test. Samples with few pneumococci, or which otherwise give poor results by this test, may be inoculated intraperitoneally into a mouse, and the mouse's peritoneal washings used 6 to 18 hours later for this or other typing effort. Specimens of spinal fluid and cultures in blood or serum broth may also be typed directly.

b. **MATERIALS.** The following materials are necessary:

- (1) Platinum loop (1 mm) for transferring sputum.
- (2) Platinum loop (4 mm) for transferring serum and dye.
- (3) Loeffler's methylene blue (not required if dye is already present in the typing serum).
- (4) Glass slides and cover glasses.
- (5) Typing sera (rabbit); types I to XXXIII monovalent and group mixtures — A (types I, II, VII; B (III, IV, V, VI, VIII); C (IX, XII, XIV, XV, XVII, XXXIII); D (X, XI, XIII, XX, XXII, XXIV); E XVI, XVIII, XIX, XXI, XXVIII); F (XXIII, XXV, XXVII, XXIX, XXXI, XXXII). These sera may be available in capillary tubes, each with enough for one test, or in small (1 ml) bottles.

c. **TECHNIC OF TEST.**

- (1) Divide three clean slides in halves with a wax pencil, and label the halves A, B, C. D. E, and F.
- (2) Place a tiny fleck of sputum in the center of an area on a slide, using the small platinum loop.
- (3) Add the typing serum, using the large loop, or express it from a capillary tube.
- (4) Add a large loopful of methylene blue (unless dye is already present in the serum).
- (5) Mix the materials on the slide thoroughly and apply a cover glass.
- (6) Let the preparation stand for 5 minutes (prepare other slides while waiting).
- (7) Examine under the oil-immersion objective for dark blue diplococci surrounded by unstained areas with definite outlines (swollen capsules). Only small indistinct capsules are seen around pneumococci mixed with heterologous antisera; large distinct halos surround pneumococci mixed with the homologous antiserum. If none of the group mixtures are found positive at first examination, yet pneumococcus-like organisms were seen in a stained smear, reexamine the typing preparations from time to time over a period of 30 minutes.
- (8) A positive reaction having been found in one of the group mixtures, repeat the test with each type of serum represented in that mixture until the positive type or types have been determined.

Caution: A certain amount of danger to the technician is involved in performing Neufeld tests, owing to the possibility of

tuberculosis infection, particularly in civilian hospitals. In some laboratories, lantern-slide cover glasses are used in place of ordinary slides. These permit the placing of six preparations under ordinary cover glasses, and still provide sufficient margin so that the fingers of the technician are not contaminated by sputum while examining the slide. If ordinary slides are used, the technicians should appreciate the dangers of auto-infection and act accordingly.

305. Meningococcus Grouping

a. GENERAL.

- (1) The meningococci are divided antigenically into groups I, II, II alpha, and IV. Organisms of groups I and II alpha are encapsulated and are clinically significant. Group I meningococci are especially prominent in epidemic outbreaks of meningitis. Group II organisms are common in the normal respiratory tract. Grouping antisera (rabbit) are available for all but the medically unimportant group IV, and a polyvalent horse serum is supplied which contains antibodies for groups I, II, II alpha, and IV.
- (2) Freshly isolated meningococci may be grouped with the sera discussed in (1) above by means of a slide agglutination, a tube agglutination or a *Quellung* test. The slide agglutination test is rapid and conserves serum. This test is performed as indicated in paragraph 302. Questionable results should be checked by the tube test (see *b* below). In fresh spinal fluid, organisms of groups I and II alpha may be identified by a direct *Quellung* test. The procedure is the same as that used for pneumococcus typing.
- (3) Normal horse or rabbit serum, if available, should be used to determine if the organism is sensitive to normal serum.

b. PROCEDURE FOR GROUPING BY THE TUBE TEST. The test is carried out as described in the procedure for the macroscopic agglutination test (par. 299), with the following modifications:

- (1) Use eight tubes or less, according to titer of serum (seven serum dilutions and one antigen control).
- (2) For the first tube use 0.8 ml of saline and 0.2 ml of serum. It is important that the final mixture have a pH not above 7.0, as high pH values will prevent agglutination. Buffer solution of pH 6.6 (see table IX) is con-

venient for making serum dilutions as well as for suspensions of organisms. Prepare serial serum dilutions in the remaining tubes as indicated in paragraph 299.

- (3) A living antigen is prepared by suspending the growth from a 16- to 24-hour slant culture of the unknown organism in normal saline. This should be accomplished by rolling the tube gently between the hands. No particles of agar should be included in the suspension. A wire loop or moistened swab may be used to loosen the growth from the slant. Dilute the suspension with normal saline solution until the turbidity corresponds to that of tube No. 3 of the MacFarland scale.
- (4) Add 0.5 ml of the antigen suspension to each tube of the diluted sera and to the antigen control. The final serum dilution in the first tube will be 1:10. Since a living antigen is being used, the tops of the tubes should be flamed before being incubated, and preferably plugged with cotton.
- (5) Shake the tubes vigorously for a minute, and place them in a water bath at 37° C. After 2 hours read, record, and place in a refrigerator. Read the final results on the following morning.

c. **SMALL LABORATORIES.** These laboratories should use only the polyvalent serum. If it is necessary to determine the group of the organism, a culture should be forwarded to the proper army area medical laboratory or general medical laboratory, where the group will be determined.

306. *Salmonella* and *Shigella* Grouping and Typing

a. "Species" of *Salmonella* are identified on the basis of an analysis of their somatic (O) and flagellar (H) antigens. A simplified kit and instructions are available. The Communicable Disease Center of the U.S. Public Health Service has published (CDC Bulletin, May 1950) an excellent description of simplified serologic identification of *Salmonella* cultures. The following brief description is intended to promote an understanding of the procedure:

- (1) An O antigen is prepared from the unknown culture by suspending the growth from an agar slant in 1 ml of absolute alcohol. This is heated at 60° C. for 1 hour, centrifuged, and suspended in 0.5 ml of physiological saline solution containing 0.5 percent phenol. Using the macroscopic spot agglutination technic, the antigen is tested against 26 basic O antisera and then against

absorbed single factor O antisera. This places the strain in a specific O group.

- (2) For determining the H factors, a young, motile broth culture, killed by the addition of an equal volume of physiological saline solution containing 0.6 percent, formalin, is used. To 1-ml portions of this antigen are added 0.02-ml amounts of the possible or suspected H antisera. The tubes are incubated in a water bath at 50° to 52° C., and read at intervals up to 2 hours. A typical floccular, flagellar type of agglutination usually occurs in 30 minutes. By use of the basic H antisera and absorbed single factor H antisera, the flagellar or H antigens, both phase 1 and phase 2, are determined. This procedure is rapid and extremely valuable for epidemiological studies, especially those related to food poisoning outbreaks due to species of *Salmonella*. The antigenic components of a few of the commoner species and strains are shown in table XXVI on page 400.

b. The *Shigella* group also is classified on the basis of somatic antigens and a simplified kit for grouping is available. See reference in *a* above for a detailed description of methods used. A brief description is given in paragraph 352 and table XXXIII lists the antigenic types by groups.

307. Streptococcus Grouping and Typing

The classification of streptococci is incomplete without consideration of their antigenic components. To date, such serological studies have been confined largely to the beta-hemolytic streptococci. By the precipitin technic of Lancefield these may be assigned to a number of serological groups, designated by letters A, B, C, D, E, F, G, H, and L. Within these groups a number of types may be distinguished. Types are designated by numbers, 1, 2, 3, etc. Beta-hemolytic streptococci of groups A, C, and G are responsible for most of the acute streptococcal infections of man. Those of group A are usually responsible for epidemic infections. Members of group C cause most epidemic streptococcal infections in animals and may spread to humans. Those other than group A are not uncommonly harbored in the human throat, intestine, and genital tract. Group D streptococci include enterococci, although most of the latter are nonhemolytic. The serological grouping of streptococci, therefore, sometimes assumes major diagnostic and epidemiologic significance. As there are many types within group A, the determination of type is important epidemiologically.

a. GROUPING. Grouping requires the use of specific grouping antisera and antigens extracted from the streptococci. Directions for the Lancefield method of extracting the group polysaccharide (by treating with hydrochloric acid) accompany the antisera sets which are supplied to qualified laboratories. A rapid and simplified procedure (Maxted) employs the proteolytic activity of a filtrate of *Streptomyces* to obtain a clear polysaccharide extract.

Table XXVI. Partial Kauffman-White Schema of *Salmonella* Classification Showing Typical Members of Each Group and Those Most Commonly Encountered*

Group and components	O antigen	H antigens	
		Phase 1	Phase 2
Group A:			
S. paratyphi A	[II], II, XII...	a	
Group B:			
S. paratyphi B	[I], IV, [V], XII...	b	1,2...
S. typhimurium	[I], IV, [V], XII...	i	1,2,3...
S. san diego	IV, [V], XII...	e,h	e,n,z ₁₄ ...
S. saint paul	I, IV, V, XII...	e,h	1,2,3...
S. abortus equi	IV, XII...		e,n,x...
S. bredeney	I, IV, [XXVII], XII...	l,v...	1,7...
Group C:			
S. paratyphi C (hirschfeldii)	VI, VII...[VI]	c	1,5...
S. choleraesuis	VI, VII...	c	1,5...
S. oranienburg	VI, VII...	m,t	
S. bareilly	VI, VII...	y	1,5...
S. montevideo	VI, VII...	g,m,s...	
S. newport	VI, VIII...	e,h	1,2,3...
S. kottbus	VI, VIII...	e,h	1,5...
Group D:			
S. typhi	IX, XII...[VI]	d	
S. enteritidis	[I], IX, XII...	g,m	
S. panama	I, IX, XII...	l,v...	1,5...
S. javiana	[I], IX, XII...	l,z ₂₄ ...	1,5...
Group E:			
S. give	III, X, XXVI	l,v...	1,7...
S. uganda	III, X, XXVI	l,z ₁₃ ...	1,5...
S. anatum	III, X, XXVI	e,h	1,6...
S. newington	III, XV	e,h...	1,6...
S. senftenberg	I, III, XIX	g,s,t...	
Further groups:			
S. rubislaw	XI	r	e,n,x...
S. worthington	I, XIII, XXIII	l,w...	z...
S. onderstepoort	(I), VI, XIV, XXV	e,(h)	1,5...
S. ballerup	XXIX [VI]	z ₁₄	

*Certain of these names do not agree with officially accepted rules of nomenclature but are terms in general use.

[] These antigens may be present.

() Only a part of these antigens are present.

... Abbreviated formulae.

The technic of the recommended capillary tube method of testing for precipitation in the presence of antisera also accompanies the antisera sets.

b. **TYPING.** The group A beta-hemolytic streptococci are divided into more than 40 types on the basis of the so-called "M" protein substances. These are extracted from the streptococci by the same Lancefield method used for extracting the group polysaccharides, and the test is similarly performed.

c. **AVAILABILITY OF ANTISERA.** The supply of antisera for both grouping and typing is limited. Designated large laboratories with experienced personnel will be supplied with these antisera and all cultures for grouping and typing should be forwarded to them.

308. Heterophile Antibody Test for Infectious Mononucleosis

a. **GENERAL.** The sera of patients with infectious mononucleosis often contain heterophile antibodies which agglutinate sheep erythrocytes. Heterophile antibodies are also produced by the injection of horse serum especially if serum sickness results. The antibodies produced by horse serum are absorbed by guinea pig kidney while the antibodies in infectious mononucleosis are not absorbed. Beef cells absorb both types of antibodies but not those found in normal sera. This is the basis of Davidsohn's differential test.

b. PRESUMPTIVE TEST FOR INFECTIOUS MONONUCLEOSIS.

- (1) Prepare a 2 percent suspension of washed sheep corpuscles in physiological saline solution. The sheep blood must be 24 hours old but not over 1 week old, and the cells should be washed three times in physiological saline solution. The supernatant fluid should be clear after the third washing.
- (2) Inactivate the patient's serum by heating at 56° C. for 30 minutes.
- (3) Place 12 small test tubes in a rack and place 0.4 ml physiological saline solution in the first tube and 0.25 ml in each of the remaining tubes.
- (4) Add 0.1 ml of serum to the first tube; mix and transfer 0.25 ml to the second tube and so on to the eleventh tube from which 0.25 ml is discarded after mixing. The twelfth tube is the corpuscle control.
- (5) Add 0.1 ml of 2 percent suspension of washed sheep cells to all tubes.
- (6) Mix well and read after 2 hours at room temperature.
- (7) Shake the tubes to suspend the sedimented cells and note clumping. The highest dilution in which clumping is

detected, either with the naked eye or the low power of the microscope, is taken as the end point. A titer of 1:224 or higher is presumptive evidence of infectious mononucleosis.

- (8) The final dilution in the first tube is 1:7, in the second tube 1:14 and so on to 1:7168 in the eleventh tube.

c. **DIFFERENTIAL TEST FOR INFECTIOUS MONONUCLEOSIS.** If the presumptive test yields a titer of 1:224 or higher the differential test may be performed as a further diagnostic aid.

- (1) Guinea pig kidney. Prepare a 20 percent suspension of mashed guinea pig kidney in physiological saline solution. Guinea pig kidneys may be kept frozen in the refrigerator till needed, then thawed, cut into slices, and washed in physiological saline solution till free of blood. Mash in a mortar and prepare a 20 percent suspension in saline. The 20 percent suspension is boiled for 1 hour on the water bath and lost fluid is replaced with distilled water. Add phenol to make a 0.5 percent solution and the suspension will keep in the refrigerator many months.
- (2) Beef cells. Wash beef erythrocytes three times in physiological saline solution and make a 20 percent suspension of packed cells in physiological saline solution. Boil for 1 hour on the water bath and replace fluid lost by evaporation with distilled water, add phenol to make a 0.5 percent solution and keep in the refrigerator.
- (3) Place 1 ml of the guinea pig kidney suspension in one test tube and 1 ml of the beef cell suspension in another tube.
- (4) Add 0.2 ml of inactivated patient's serum to each tube and let stand at room temperature for 1 hour shaking at 15 minute intervals.
- (5) Centrifuge at 1,500 rpm for 10 minutes and remove the supernatant fluid with a pipette. This is a 1 to 5 dilution of serum.
- (6) Repeat the presumptive test as described above using the absorbed serum and add 0.25 ml of this 1 to 5 dilution to the first and second tubes. No saline is placed in the first tube but the other tubes contain 0.25 ml of saline as in the presumptive test. Mix and transfer 0.25 ml from the second tube to the third tube and so on through the eleven tubes, discarding 0.25 ml from the eleventh tube. The twelfth tube is the corpuscle control.

d. **INTERPRETATION.**

- (1) The guinea pig kidney does not absorb the agglutinins of infectious mononucleosis but does absorb those due to

horse serum and also anti-sheep agglutinins normally found in serum. If the titer before absorption with guinea pig kidney is 1:224, the titer after absorption will be not less than one-fourth of the original titer or 1:56. A titer lower than 1:56 after absorption would be against a diagnosis of infectious mononucleosis.

Beef cells absorb the agglutinins of infectious mononucleosis and those due to horse serum but not those normally present in serum.

(2) Agglutinins for sheep erythrocytes in human serum.

Type of serum	Absorbed by guinea pig kidney	Absorbed by beef cells
Normal -----	+	—
Infectious mononucleosis—	—	+
Serum sickness-----	+	+

309. Cold Agglutinins

a. GENERAL. Demonstration of cold agglutinins is of diagnostic importance in virus (atypical) pneumonia but a negative test does not invalidate the diagnosis.

b. MATERIALS FOR COLD HEMAGGLUTININ TEST.

- (1) Kahn tubes and racks.
- (2) Serological pipettes, 1.0 ml graduated in hundredths.
- (3) 0.85 percent NaCl solution.
- (4) Cells. Sterile human group O blood is used. Draw 20 ml from a healthy adult and add to an equal volume of Alsever's solution. Alsever's solution contains 2.05 percent dextrose, 0.42 percent sodium chloride, 0.8 percent trisodium citrate, and 0.55 percent citric acid. Twenty ml amounts of solution are distributed in 50 ml vaccine bottles; air is partially removed by suction applied through a needle inserted through the rubber stopper, and the evacuated bottle is autoclaved at 15 pounds for 15 minutes. A supply of bottles of solution may be kept on hand. Freshly drawn blood while still in the syringe is injected through the stopper, which has been cleaned with an iodine swab, and mixed with the sterile Alsever's solution. These sterile blood mixtures may be stored at 5° C. for 2 weeks and portions removed aseptically when

needed to prepare suspensions of washed erythrocytes. The cells are washed three times by sedimentation in the centrifuge and resuspended in 10 volumes of 0.85 percent NaCl solution. The final centrifugation is done in a graduated 15 ml tube at 2,000 rpm for 10 minutes, the supernatant fluid is pipetted off, and the volume of packed cells determined by inspection. Sufficient 0.85 percent NaCl solution is added to make a 1.0 percent suspension of erythrocytes. Usually 5 ml of blood-*Alsever's* solution mixture yields about 1.2 ml of packed cells. Washed red cells are unsatisfactory after storage and any residue from the day's tests should be discarded. The patient's own cells may be used for the test cells if group O blood is not readily available.

- (5) Patient's serum. Cold hemagglutinins are absorbed by erythrocytes at refrigerator temperature. Therefore, handle blood for cold hemagglutinin tests with care. Venous blood should be allowed to clot at room temperature, centrifuged, and the serum removed from the clot, and then stored in the refrigerator.

c. PROCEDURE FOR TEST.

- (1) To each of a series of 10 Kahn test tubes is added 0.3 ml of 0.85 percent NaCl solution; 0.3 ml of the patient's serum is added to the first tube, mixed, and 0.3 ml removed and added to the second tube, etc. In this manner serum dilutions covering the range from $1/2$ to $1/1024$ are prepared.
- (2) 0.3 ml of a 1 percent suspension of washed "O" cells is added to each tube.
- (3) The tubes are shaken and placed overnight in a refrigerator maintaining a temperature of 0° to 5° C. It is important that the temperature remain within this range. Upon removal from the refrigerator next morning, the tests should be read promptly since disaggregation may occur quickly at room temperature.
- (4) For reading, each tube is held in a good light and the bottom is flicked; if complete aggregation has occurred, a disk of erythrocytes floats up from the bottom of the tube; this is a 4 plus reaction. Tubes showing lesser degrees of agglutination are rotated back and forth while held vertically between the palms and then read with the aid of a hand lens. A titer of 1-64 or higher is of diagnostic significance.

- (5) In order to be sure that the aggregation is caused by cold hemagglutinins, the test tubes are next transferred to a 37° C. water bath and reread 2 hours later. The agglutination should have disappeared.

Section VII. HANDLING OF BACTERIOLOGICAL SPECIMENS

(See also Section I, Collection of Bacteriologic Specimens)

310. General

The following directions are suggested as a guide to be used in handling specimens submitted for bacteriological examination. It must be remembered that it is not a complete list of possibilities, and only organisms commonly encountered are given consideration.

311. Blood for Culture

a. Among the organisms encountered are: *Salmonella* types including *S. typhi* (typhoid), *Brucella*, *Streptococcus*, *Staphylococcus*, pneumococcus, meningococcus, *Hemophilus influenzae*, and *Pasteurella pestis*.

b. Direct smears of the blood are of no value.

c. Culture blood, taken aseptically, in an appropriate broth or semisolid medium (2 to 5 ml per 100 ml medium). Poured plates containing 1 ml of blood should be made. Incubate cultures aerobically, anaerobically, or in an atmosphere containing 2 to 10 per cent carbon dioxide, as indicated. (See par. 272*i* and *j*.)

312. Blood for Serological Study

a. Serum is obtained as directed in paragraph 227.

b. The serum may be used for Widal, Weil-Felix, and similar test.

c. Antibodies are not produced at once. Blood for serological studies should never be taken before the fifth or sixth day of illness and preferably after 10 to 12 days, unless early specimens are desired to establish a base for detecting a subsequent increase in titer of specific antibodies.

313. Bile

a. The usual organism encountered in *Salmonella typhi* (typhoid).

b. Direct smears are of doubtful value, but a gram stain should be made.

c. Inoculate selenite enrichment broth as well as differential plate media (see par. 272a to e).

d. If a pyogenic infection is present, use blood agar plates, aerobic, and anaerobic.

314. Feces

(See par. 349.)

a. The most common pathogens encountered are *Salmonella* types, including *S. typhi* (typhoid), *Shigella* (dysentery), and *Vibrio* (cholera).

b. Direct smears are of value in determining the presence of cellular constituents (use differential stains, for example, Wright's stain, or hematoxylin and eosin). In cholera and shigelloses sufficient information can be obtained from a direct examination to give a presumptive diagnosis.

c. Culture on differential plate media (EMB agar, SS agar, desoxycholate-citrate agar, alkaline agar, etc.). (See par. 272a, b, c, and f.)

d. Culture in enrichment medium (Selenite-F broth), with subsequent subculturing on differential media. (See par. 272d, e, and f.)

e. Pick suspicious colorless colonies found on selective media. Inoculate Kligler's iron agar or triple sugar iron agar and incubate for 24 hours. Select colonies and interpret the results in accordance with table XXX. See paragraph 349 for further procedure.

f. If cholera is suspected, proceed in accordance with paragraph 359.

315. Urine

a. The organisms encountered include *Salmonella typhi* (typhoid), *Salmonella* (paratyphoid), *Proteus*, *Escherichia coli*, *Aerobacter*, *Mycobacterium tuberculosis*, *Streptococcus*, *Staphylococcus*, and *Neisseria gonorrhoeae*. (See par. 232.)

b. Concentrate specimen by centrifugation.

c. Make a direct smear; stain by the gram or acid-fast method, or by both.

d. Culture on EMB agar, blood agar (aerobic and anaerobic), or chocolate agar, as indicated. Inoculate selenite-F and special purpose broth. (See par. 272d and i.) After overnight incubation streak the broths on appropriate plate media.

e. If tuberculosis is suspected, culture on an appropriate medium or inject the concentrated sediment into a guinea pig, intramuscularly, or both.

316. Sputum

a. The organisms encountered include pneumococcus, *Mycobacterium tuberculosis*, *Streptococcus*, *Staphylococcus*, *Hemophilus influenzae*, and *pertussis*. Rarely found are *Fusobacterium* and *Borrelia* (Vincent's), *Klebsiella* (Friedländer), and fungi.

b. Examine a direct smear stained by the gram or acid-fast stain, or both if indicated.

c. If tuberculosis is suspected, concentrate the sputum and then proceed as follows:

- (1) Make an acid-fast stain.
- (2) Inoculate appropriate media.
- (3) Inject a guinea pig, when requested.

d. If pneumonia is suspected, proceed as follows:

- (1) If pneumococci are present in large numbers, set up a Neufeld test with pooled antisera.

(a) If the test is positive, identify the type using specific antisera.

(b) If test is negative:

1. Prepare blood agar cultures to detect the presence of *Streptococcus*, *Staphylococcus*, etc.
2. Inject a mouse intraperitoneally, for subsequent typing of the peritoneal washings.

- (2) If pneumococci are not present in large numbers, inject a mouse intraperitoneally for subsequent typing, and inoculate a blood agar or chocolate agar plate or both, incubating aerobically, anaerobically, and in increased carbon dioxide.

317. Cerebrospinal Fluid

a. The organisms encountered include *Neisseria* (meningococci), pneumococcus, *Hemophilus influenzae*, *Streptococcus*, *Mycobacterium tuberculosis*, and, rarely, *Salmonella*, *Staphylococcus*, and *Torula*.

b. Prepare a direct smear of the centrifuged sediment or from coagulum that may form. Stain by the gram or acid-fast method, or both.

c. Culture the centrifuged sediment on blood agar and chocolate agar plates aerobically and in an atmosphere containing an increased amount of carbon dioxide.

- d. If tuberculosis is suspected, culture on an appropriate medium or inoculate a guinea pig, or both.
- e. If yeastlike cells are seen, culture on Sabouraud's agar.

318. Pus From Wounds, Boils, Peritonitis, etc.

a. The organisms encountered include *Staphylococcus*, *Streptococcus* (aerobic and anaerobic), *Escherichia coli*, *Aerobacter aerogenes*, *Clostridium* (gas-gangrene anaerobes), *Proteus*, *Pseudomonas* (pyocyaneus), and many others.

b. Examine a direct smear stained by the gram method (support by a spore stain, if needed).

c. If coccus forms alone are present, culture on blood agar plates (aerobic and anaerobic).

d. If gram-negative rods alone are present, grow on plain agar plates, differential media (EMB agar, SS agar, etc.) and on blood agar plates. Look for pigment production.

e. If spore formers are present, culture on blood agar plates (aerobic and anaerobic), inoculate milk and seal with paraffin-vaseline (to test for "stormy fermentation," and inoculate other appropriate specialized anaerobic media, such as thioglycollate broth, Robertson's cooked-meat medium, etc.

f. From areas contaminated by fecal material, such as abdominal wounds following a ruptured appendix or perforation of the intestine, the direct smear usually shows many types of organisms. Inoculate various selective media, such as EMB agar, desoxycholate-citrate agar, SS agar, blood agar plates (aerobic and anaerobic), thioglycollate medium, etc. Where there is such a variety of organisms, solid media are generally preferable, since in broth the more significant pathogens are likely to be overgrown by such ubiquitous organisms as *Proteus vulgaris* and *Escherichia coli*. Utilize the selective bacteriostatic effects of chloral hydrate, desoxycholate, tellurite, etc. (See par. 270i.)

319. Pleural, Pericardial, or Other Fluids From Serous Cavities

a. These are often sterile. It may be necessary to add an anticoagulant as soon as the fluid is drawn to prevent it from solidifying. The organisms encountered include *Streptococcus*, pneumococcus, *Staphylococcus*, *Mycobacterium tuberculosis*.

b. Centrifuge, and from the sediment prepare a gram or acid-fast stain, or both.

c. Inoculate the sediment to blood agar plates (aerobic and anaerobic).

d. If tuberculosis is suspected, inoculate appropriate media and inject a guinea pig.

320. Material From Throat and Tonsils

a. The organisms include *Streptococcus*, *Staphylococcus*, pneumococcus, *Neisseria* (meningococcus), *Corynebacterium* (diphtheria), *Fusobacterium* and *Borrelia* (Vincent's), and fungi.

b. Stain by the gram method, with Loeffler's methylene blue or another differential stain for diphtheria bacilli and with dilute carbolfuchsin for fusiform bacilli and spirochaetes (Vincent's).

c. Inoculate blood agar or chocolate agar plates, or both, as indicated (aerobic, anaerobic, and increased CO₂). For hemolytic streptococci, inoculate blood broth containing sodium azide. (See par. 270i.)

d. If diphtheria is suspected, inoculate selective tellurite medium or tellurite medium and Loeffler's medium. (See par. 272g and h.)

321. Materials From Urethra and Prostate

a. The usual organism encountered is *Neisseria gonorrhoeae*.

b. Examine a direct smear stained by the gram method.

c. Inoculate warm chocolate agar plates at once and incubate under increased CO₂.

d. The oxidase reaction will assist in recognizing and isolating colonies.

322. Materials From Ear and Mastoid

a. The organisms encountered include *Streptococcus*, *Staphylococcus*, and pneumococcus.

b. Study a direct smear stained by the gram method.

c. Inoculate blood agar plates (aerobic and anaerobic).

323. Materials From Nose, Sinuses, and Nasopharynx

(See par 320)

a. The organisms encountered include *Streptococcus*, *Staphylococcus*, *Neisseria* (meningococcus), *Corynebacterium*, *Hemophilus influenzae*, *H. pertussis*, and pneumococcus.

b. Examine a direct smear stained by the gram method or with Loeffler's methylene blue, if diphtheria is suspected, or both.

c. Culture on blood agar plates (aerobic and anaerobic), chocolate agar plates (increased CO₂), Loeffler's serum slants, etc., as indicated.

324. Materials From Eye

a. The organisms encountered include *Streptococcus*, *Staphylo-*

coccus, pneumococcus, *Neisseria* (gonococcus), *Corynebacterium* (diphtheroids), *Hemophilus influenzae*, *H. duplex*, and fungi.

- b. Examine a direct smear stained by the gram method.
- c. Culture on blood agar, chocolate agar, or Sabouraud agar plates, as indicated.

325. Materials From Skin (for Fungi)

- a. Divide the specimen in two equal parts.
- b. Macerate one portion in 10 percent sodium hydroxide for 30 minutes or longer; then examine microscopically an unstained moist (cover glass) preparation of the material.
- c. Inoculate media in accordance with instructions given in paragraph 399.

Section VIII. PREPARATION OF AUTOGENOUS VACCINES

326. Selection of Subculture

An autogenous vaccine is one prepared from a culture isolated directly from the patient who is to be treated with that vaccine. With the infected materials, such as pus or tissues, prepare gram-stained films and examine them for the types of organisms present. Inoculate blood agar and infusion agar plates and a tube of infusion broth and incubate for 24 to 48 hours at 37° C. Examine the plates for predominating types of colonies and make gram-stained preparations from each type. Also examine stained preparations from the broth culture for presence of any organism not found on plate cultures and inoculate upon plate media if indicated. If an organism is found in pure culture, transplant to the desired medium for the vaccine. When mixed cultures are obtained, select isolated colonies of the type desired and transfer to plates to isolate pure cultures; incubate for growth and again check for purity of cultures. If several organisms are present, presumptive evidence as to which organism is concerned in the infection can sometimes be gained, first, by agglutination of the organism by patient's serum, or, second, by a positive skin reaction on intradermal injection of the vaccine. Do not use spore-forming bacilli or any organisms that are obviously contaminants. Staphylococci and Streptococci are the organisms most frequently used for the production of autogenous vaccines.

327. Preparation of Suspensions

- a. AGAR SLANT CULTURES. Most bacteria are best grown on infusion agar slants for 24 to 48 hours; blood or serum agar is

required for growing more fastidious organisms. Add 2 or 3 ml of sterile saline to each tube and emulsify the bacteria by shaking or by agitation with a platinum loop. The suspension should be quite heavy. If any clumps are observable in the bacterial suspension, transfer to a sterile flask or bottle containing beads, and shake thoroughly; then filter aseptically through several layers of gauze, held in a small funnel, to break up the remaining clumps and to remove any particles of culture medium.

b. **BROTH CULTURES.** If broth cultures are used, sediment the bacteria by centrifuging and resuspend in sterile normal saline solution; centrifuge a second time and resuspend in sufficient saline solution to give a heavy suspension. After the clumps of bacteria have been broken up, continue as above.

328. Determining Bacterial Content

a. **WRIGHT'S METHOD.** Prepare a capillary pipette with a long capillary section and place a mark about 1 cm from the tip. Draw up blood from the fingertip to the mark, then a small air bubble, and then bacterial suspension to the same mark. Mix quickly on a slide, make thin smear, and stain by Wright's method. Count the number of red cells and the number of bacteria in several areas. Use the following formula to determine the number of bacteria per milliliter of suspension:

$$\frac{\text{Bacteria counted}}{\text{Red cells counted}} \times 1,000 \times 5,000,000 = \text{number of bacteria per milliliter.}$$

b. **NEPHELOMETRIC METHOD (MACFARLAND).** This method, employable when the suspension contains no coloring matter, consists in comparing the opacity of the bacterial suspension with that of various densities of barium sulfate in a series of test tubes.

- (1) *Preparation of standards.* Prepare 1 percent aqueous solutions of chemically pure sulfuric acid and barium chloride. To a series of 10 Pyrex test tubes of uniform size add increasing amounts of the barium chloride solution, starting with 0.1 ml in first tube, and increasing the quantity by 0.1 ml in each succeeding tube to 1.0 ml in the tenth tube. Then add to each tube enough of the acid solution to bring the total volumes to 10 ml (9.9 ml to 9.0 ml, respectively). Seal hermetically in a Bunsen or blowtorch flame, and label serially from 1 to 10. The densities of the suspensions in these tubes correspond approximately to 300 million organisms per millimeter in the first tube, and increasing by 300 million bacteria for each succeeding tube to 3,000 million per milliliter in the tenth tube.

- (2) *Technic.* Place a measured quantity (1.0 ml or more, depending on the density) of the bacterial suspension in a test tube of the same diameter and color as those used for the standard. Dilute by adding a measured amount of sterile saline solution to the density of one of the standards; shake well during the process.
- (3) *Calculation.* The approximate number of bacteria per milliliter of suspension corresponds to the tube matched, times the dilution. For example, if 1.0 ml of suspension was diluted to 4 ml to match tube No. 4, it contains $1,200,000,000 \times 4$, or 4,800,000,000 organisms per milliliter.

329. Preparation of Vaccine

Most autogenous vaccines for treatment are prepared in a concentration of 1 billion organisms per milliliter. To prepare a definite quantity, say 30 ml of vaccine of this strength from the bacterial suspension above, find the number of milliliters of suspension required by use of the following formula:

$$\frac{30 \text{ (ml of vaccine)} \times 1,000,000,000 \text{ (strength of vaccine)}}{4,800,000,000 \text{ (strength of suspension)}} = 6.25 \text{ ml}$$

After standardization, add phenol to a final concentration of 0.5 percent (1 ml of a stock 5 percent phenol solution to 9 ml of vaccine) for preservation. The bacteria are dispensed in a properly labeled, sterile vaccine bottle, closed with a rubber stopper, and killed as indicated in paragraph 330.

330. Killing of Bacteria

Weight the bottle with sheet lead, and immerse in a water bath at 56° to 60° C. for 1 hour. Remove from the water bath and culture for sterility by inoculating at least four 0.25-ml portions into tubes containing at least 15 ml of fluid thioglycolate medium, incubating for 7 days.

Section IX. OTHER TYPING AND TESTING PROCEDURES

331. Bacteriophage Typing of Bacteria

The susceptibility of certain groups of bacteria to highly specific strains of bacteriophages is finding increasingly wider use in the epidemiological differentiation of strains. Thus, specific bacteriophages have been developed for many strains of typhoid bacilli possessing Vi antigen, as well as for strains of *S. paratyphi B*, *Staphylococcus aureus*, and others. With these reagents it is

possible to differentiate strains not separable by other methods. When circumstances indicate a need for specific phage typing, cultures and information should be forwarded to the Army Medical Service Graduate School.

332. Antibiotic Discs for Sensitivity Testing

Discs of filter paper containing measured amounts of an antibiotic provide a convenient method for determining the sensitivity of bacteria to chloramphenicol, bacitracin, penicillin, or streptomycin. The discs are distributed on the surface of agar which has been inoculated with the organism to be studied. Directions for using and for interpreting the results of tests are given with the discs.

CHAPTER 8

CLASSIFICATION OF BACTERIA AND FUNGI

Section I. DESCRIPTION OF BACTERIA

GRAM-POSITIVE COCCI

*The names *Micrococcus pyogenes* var. *aureus* and *M. pyogenes* var. *albus* are used in the sixth edition of Bergey's Manual of Determinative Bacteriology, for *Staphylococcus aureus* and *albus*. The shorter and more familiar older names are retained in this manual.*

333. *Staphylococcus Aureus*

a. CHARACTERISTICS. Staphylococci are spherical organisms appearing in grapelike clusters, particularly when grown on solid media. Diplococcoid forms and occasional short chains will also often be seen in fluid media and indirect smears from pus. Growth is abundant on ordinary nutrient media. On blood agar, after 24 hours' incubation, the surface colony is 3 to 4 mm in diameter, raised, glistening, and opaque; hemolysis usually occurs, but deep colonies are often nonhemolytic. The characteristic ivory to golden yellow pigment may develop slowly; it will become more marked if the culture is left at room temperature after the initial incubation at 35° C. The development of pigment is enhanced when the culture is grown on 10 percent milk agar (10 ml of evaporated milk to 90 ml of nutrient agar) and this medium should be used to check lack of pigment formation. Loeffler's medium also may be used, but milk agar is preferred. Some variation in pigment occurs, thus white variants may be found in pure cultures of typical *aureus* cultures. Gelatin is usually liquefied after 2 to 3 days. Toxin-producing, pathogenic strains usually ferment mannitol and are coagulase positive. The coagulase test (par. 295) is considered to be more closely correlated with pathogenicity. Resistance to heat and chemical disinfectants is greater than that of the average bacterium; many strains survive 60° C. for 30 minutes. Staphylococci grow very abundantly in the presence of 5 to 10 percent sodium chloride and media containing 8 to 10 percent sodium chloride is widely recommended for selective isolation of staphylococci. Employing this characteristic, combined with the tendency to ferment mannite, a selective differential medium for staphylococci may be prepared by adding 2 gm of

agar, 8 gm of sodium chloride and 1 gm of mannite to each 100 ml of phenol red broth base (par. 274a(3)). Colonies of mannite-fermenting staphylococci are surrounded by yellow zones. Dissociation of cultures maintained in salt agar is reduced and coagulase-production is enhanced. (Jour. Bact. 50:201, 1945.)

b. HOST RELATION. *Staph. aureus* is the commonest cause of severe localized purulent infections in man. The lesion may remain local or it may spread by direct extension, or by the blood stream, giving metastatic lesions in almost any part of the body; it may thus appear in blood cultures. A necrotizing exotoxin produced by the organism is apparently responsible for a large part of the damage it produces. In addition, the organism is frequently implicated in a toxic type of food poisoning following the ingestion of food contaminated with it. The enterotoxin appears to be a polysaccharide and withstands prolonged boiling, hence food containing it may produce symptoms although sterile from cooking. Although kittens are susceptible to the enterotoxin when it is produced in special media, no method of detecting it in food is yet available, except by feeding human volunteers. (See also par. 403c.)

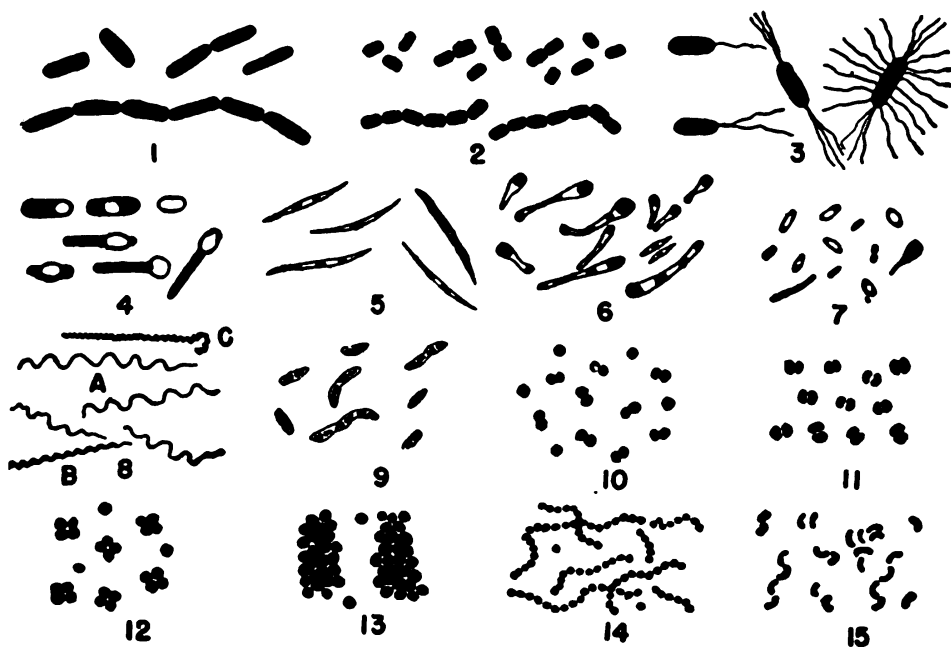
c. DIAGNOSTIC CRITERIA. The demonstration of characteristic gram-positive cocci appearing in clusters in connection with a relatively large opaque ivory to golden-yellow colony on blood agar is sufficient basis for the bacteriological diagnosis. As mentioned above, most pathogenic strains ferment mannitol and give a positive coagulase test.

334. *Staphylococcus Albus* and *Citreus*

a. CHARACTERISTICS. The above description of *Staph. aureus* applies to these organisms as well, except that the colonies of *Staph. albus* are white and those of *Staph. citreus* are yellow. Usually, but not always, they fail to cause hemolysis, liquefy gelatin at a much slower rate, if at all, and give a negative coagulase test.

b. STAPH. ALBUS. This organism is relatively nonpathogenic. It is associated with superficial skin infections, such as pimples and stitch abscesses, and is commonly present in normal skin. Few strains, if any, produce toxin.

c. STAPH. CITREUS. This organism is frequently found as an air contaminant. It produces lemon-yellow colonies, liquefies gelatin very slowly, does not produce coagulase and usually is non-hemolytic.



- 1 and 2. Long and short bacilli, singly, in pairs, and short chains.
3. Types of flagellated bacilli.
4. Various sizes, shapes, and positions of bacterial spores.
5. Fusiform bacilli.
6. *C. diphtheriae*, banded forms and polar bodies.
7. *Past. pestis*, bipolar staining rods and involution forms.
8. a, *Borrelia*. b, *Treponema*. c, *Leptospira*.
9. *D. pneumoniae*, encapsulated.
10. Diplococci.
11. Biscuit-shaped diplococci of *Neisseria*.
12. Tetrads.
13. Staphylococci.
14. Streptococci.
15. *Vibrio comma*.

Figure 26. Morphology of bacteria.

335. *Streptococcus Pyogenes*

This is the species of beta-hemolytic streptococci most frequently pathogenic for man. The name *Strep. hemolyticus* is used by some authors as a synonym for *Strep. pyogenes* and sometimes as a loose term for any beta-hemolytic streptococcus.

a. CHARACTERISTICS. The chain formation of *Strep. pyogenes* is best seen in smears from cultures in fluid media. In tissue sections, in exudates, and in smears from cultures on solid media, conspicuous chain formation may be lacking. The individual cocci are often flattened against each other. There may be variation in size, and gram-negative forms often appear. Capsules, appearing as unstained halos, may sometimes be seen in smears made directly from the infected material. Growth is best in the presence of blood or serum and may not take place in the usual nutrient media. The 24-hour surface colony on blood agar is about 1 mm in diam-

Table XXVII. *Differentiation of Staphylococci from Many Other Micrococci*¹

Species	Size	Grouping	Pigment	Blood agar colonies	Gelatin ² liquefaction	Mannitol ³ fermentation
<i>Staphylococcus aureus</i>	Cells relatively small.	Irregular grapelike clusters.	Ivory to golden yellow. White variants often seen.	Surface colonies usually hemolytic; deep colonies nonhemolytic.	+	A (usually).
<i>Staphylococcus albus</i> and <i>Micrococcus</i> (species)	Cells relatively small. Cells often larger than those of <i>Staphylococcus</i> .	Irregular grapelike clusters. Many have no definite arrangement in groups, others are arranged symmetrically in groups of 4 or 8.	White(<i>albus</i>); lemon yellow (<i>citreus</i>). White, yellow, or rarely pink; varies with species or strains.	Usually nonhemolytic. Usually nonhemolytic.	Usually slow. —	A (usually). —

¹ A key to the species of the genus *Micrococcus* is given in the 6th Edition of Bergey's "Manual of Determinative Bacteriology" page 236.

² The listed reactions are the usual ones; there are some exceptions.

+ = Yes.

— = No.

A = Acid formation.

eter, often nearly transparent, and either smooth, or (with drying and aging) having a collapsed and wrinkled appearance. The characteristic beta hemolysis consists of the complete destruction of the red blood cells leaving a clear transparent area around the deep colony. The extent of the hemolyzed zone varies with different strains; some will give an alpha (green) type of change as a surface colony, showing complete red cell destruction (beta hemolysis) only when under the surface of the agar. For this reason, poured blood agar plates are preferred. Frequently strains will be encountered whose surface colonies appear non-hemolytic on subsequent culture. Growth is aerobic and facultatively anaerobic. Resistance to heat and chemical disinfectants is not unusual; they will not tolerate 0.1 percent methylene blue. *Strep pyogenes* ferments lactose, salicin, and trehalose; does not ferment inulin, mannitol, and sorbitol; and does not hydrolyze sodium hippurate. Virulent strains are fibrinolytic for normal human fibrin. (See par. 294.) The above reactions are diagnostic for *Strep. pyogenes*, if the culture belongs in Lancefield's group A on the basis of the precipitin reaction. (See pars. 270i and 273.)

b. HOST RELATION. Not all beta-hemolytic streptococci are pathogenic for man. Those of the Lancefield's group A usually are pathogenic. Occasionally sporadic infections are due to certain strains of groups B, C, D, F, G, and H. *Strept. pyogenes* is responsible for infections of great diversity, perhaps the most common being throat and wound infections of varying intensity. The infections tend to spread and to invade the blood stream. Among several toxins produced is one that gives rise to the erythematous rash of scarlet fever. The presence of a rash depends on the toxigenicity of the infecting strain and the antitoxic immunity of the host.

c. DIAGNOSTIC CRITERIA. The finding of beta-hemolytic streptococci in the blood stream, in abscesses, or in closed body cavities, or the finding of relatively large numbers of them in material from infected mucous membranes, open sores, or wounds is of high diagnostic significance. Its identification and the determination of its relation to possible sources of infection can be made only after more thorough cultural and serological study. In the diagnostic laboratory the simplest, quickest, and most informative procedure is the Lancefield grouping. (See par. 307.)

d. ANTI-STREPTOLYSIN DETERMINATION. The presence of antibodies which neutralize streptolysin "O" of hemolytic streptococci is assuming considerable importance in detecting hidden infection. Requests are frequently made for anti-streptolysin determinations on sera from patients suspected of having rheumatic fever. Streptolysin or procedures for producing it and

titrating anti-streptolysin are available from the Department of Bacteriology, Army Medical Service Graduate School. This determination should not be attempted unless trained personnel are available.

336. Failure of Streptococci to Produce Catalase

Some cocci and diphtheroids closely resemble streptococci or pneumococci. Members of the tribe *Streptococceae* fail to produce catalase whereas micrococci, staphylococci and diphtheroids elaborate this enzyme. A simple and rapidly performed catalase test is given in paragraph 296.

337. Streptococcus Viridans

This name, as well as alpha-hemolytic streptococcus, is used to indicate any streptococcus forming colonies surrounded by a noticeable greenish discoloration of the blood agar. Many of these organisms are common inhabitants of the mouth and upper respiratory passages. Beta-hemolytic streptococci grown on blood agar containing dextrose also may give only greenish discoloration, and some beta-hemolytic forms may produce alpha-hemolytic variants.

a. CHARACTERISTICS. The alpha-hemolytic streptococci are chain-forming cocci, the cells often being elongated. Chain formation is best observed in fluid media. In tissue sections, in exudates, and in smears from cultures on solid media conspicuous chain formation may be lacking. Diplococcoid forms resembling pneumococci are not uncommon. Some strains show marked *pleomorphism*, with gram-negative and bacillary forms appearing. Growth is best in blood-containing media. On a blood agar plate the surface colony is 1 mm or less in diameter in 24 hours, and is usually smooth and dome-shaped, in distinction to the flat pneumococcus colony. The typical alpha hemolysis is best observed about the deep colony, and consists of a narrow zone of grayish-green red blood cells surrounded by another narrow zone in which more or less complete break-down of the red cells (true hemolysis) has occurred. The hemolysis may be intensified by refrigeration of the plate after incubation. The alpha thus differs from the beta type of hemolysis, in which complete and much more extensive cell destruction is seen, beginning immediately at the edge of the colony. In doubtful cases the type of hemolysis may be determined by examining the colonies with the low-power objective of the microscope. The alpha-hemolytic streptococci include groups of organisms whose distinguishing features are not yet clearly defined. The common inhabitants of the mouth and upper respira-

tory passages are often given the name *Streptococcus salivarius*. The majority of these are referred to by many (e.g., Bergey's Manual) as *S. mitis*, however, reserving *S. salivarius* for those producing mucoid colonies on 5 percent sucrose agar. Many enterococci (*S. faecalis*) produce alpha-hemolysis. Characteristics of this group are given in paragraph 338. Alpha streptococci frequently are isolated from the blood and heart valves of cases of subacute bacterial endocarditis. Many of these have the characteristics of *S. mitis*, and some *S. salivarius* and *S. faecalis* have been isolated from these cases. Others have been placed in a group which is termed *Streptococcus s.b.e.* (subacute bacterial endocarditis) or *S. sanguis*. They do not produce mucoid colonies on 5 percent sucrose agar, but synthesize a slime composed of dextrans in 5 percent sucrose broth. They hydrolyze arginine, usually ferment insulin and grow on 40 percent bile blood agar (J. Bact. 55; 393, 1948). These organisms have not been found in the mouth or upper respiratory passages.

b. HOST RELATION. Streptococci of this species are regularly present in large numbers in the normal throat and nasopharynx. They may be responsible for chronic, usually low-grade, infections of the tonsils, sinuses, appendix, gall bladder, and urinary tract; apical tooth abscesses are usually due to them. They are the most frequent cause of subacute bacterial endocarditis, in which the organisms localize in a previously damaged heart valve and are given off into the blood stream, often in "showers." Isolation from the blood is best attempted as the fever is rising. It is usually difficult to assign any etiologic significance to the isolation of alpha-hemolytic streptococci from the upper respiratory tract because of their constant presence in the normal person.

c. DIAGNOSTIC CRITERIA. These streptococci are identified on the basis of alpha hemolysis, lack of solubility in bile, and frequent failure to ferment inulin.

338. Enterococci

a. CHARACTERISTICS. Enterococci consist of elongated, frequently lanceolate cells. They are somewhat larger than *Strep. pyogenes* and are usually seen in short chains or pairs. Growth occurs on unenriched nutrient agar, forming colonies 1 to 2 mm in diameter in 24 hours. *Strep. faecalis*, the commonest species, produces in blood agar no visible change (gamma appearance) in the medium surrounding the deep colonies after 24 hours' incubation, possibly a slight "greening" after 48 hours, and alpha hemolysis after refrigeration overnight. (Greening is almost invariably apparent underneath a surface colony—that is, it can be seen if the

colony is scraped away with a platinum loop.) Some enterococci (*Strep. zymogenes* and *Strep. durans*) are beta hemolytic. *Strep. faecalis* ferments sorbitol and mannitol, does not ferment inulin, and is not bile soluble. The most distinctive characteristics of the enterococci are their resistance to heat (most strains surviving 60° C. for 30 minutes) and their ability to tolerate 0.1 percent methylene blue, 6.5 percent sodium chloride, and a reaction of pH 9.6. They belong to Lancefield's group D (par. 307) and are most easily identified by the streptococcus grouping technic.

b. HOST RELATION. The enterococci are regularly present in human feces. They occasionally cause urinary tract infections, and sometimes subacute bacterial endocarditis, but are more frequently present as secondary invaders in mixed infections.

c. DIAGNOSTIC CRITERIA. Identification of enterococci is based on finding rather large, frequently lanceolate, gram-positive cocci in pairs and short chains; showing either alpha, beta or no hemolysis in blood agar after 24 hours; and generally possessing the Lancefield group D polysaccharide.

339. Anaerobic Streptococci

Facultative anaerobic streptococci may acquire a complete anaerobic habit but, after isolation anaerobically, will revert and subsequently grow aerobically. Such are not true anaerobic streptococci; it is the strictly anaerobic types that are here described.

a. CHARACTERISTICS. In general, chain formation is not marked in this group; frank clumping may be seen. The individual organisms of some strains are smaller than other gram-positive cocci. The appearance of colonies in and on blood agar is somewhat influenced by the method of anaerobiasis employed; usually there is no hemolysis, but there may be brownish discoloration of the blood. Surface colonies are often about 1 mm in diameter and sometimes are brown or coalblack. Fluid thioglycollate medium is excellent for cultivating anaerobic streptococci, and isolations are often facilitated by preliminary growth in this medium. Many varieties produce an offensive putrefactive odor. Several species have been described, differentiation being based upon such characteristics as gas formation, odor, and proteolytic activity. Resistance to heat and chemicals is not unusual.

b. HOST RELATION. The anaerobic streptococci are second only to *Strep. pyogenes* as a cause of puerperal sepsis. Blood stream invasion may be expected. They are also encountered in wound infections and are normal inhabitants of the gastro-intestinal

Table XXVIII. Differentiation of *Streptococci* and *Pneumococci*

Species	Appearance of deep colonies in blood agar*	Morphology in liquid media	Capsule	Fermentation Tests				Bile solubility
				Lactose	Salicin	Mannitol	Inulin	
<i>Streptococcus pyogenes</i>	Beta.....	Usually long chains.	±	+	+	—	—	—
<i>Streptococcus salivarius</i>	Alpha.....	Usually short chains.	—	+	—	—	—	—
<i>Streptococcus faecalis</i> ¹	Gamma or alpha.....	Pairs and short chains.	—	+	+	+	—	—
<i>Anaerobic streptococci</i>	Gamma or beta (rare).....							
<i>Diplococcus pneumoniae</i>	Alpha.....	Lance-shaped diplococci or short chains.	± ²	+	—	—	+	+

* The three principal types of appearance of deep colonies in blood-agar plates are as follows:

Beta hemolysis.—The deep colony is immediately surrounded by a perfectly clear zone in which no intact red cells remain.

Alpha discoloration and hemolysis.—In the immediate vicinity of the deep colony some red cells remain intact and are frequently discolored greenish (viridans type) or brownish. In the fully developed alpha zone the intact cells are, in turn, surrounded by a zone of more or less complete hemolysis. The hemolysis is intensified by refrigeration of the plate culture.

Gamma appearance.—There is neither discoloration nor hemolysis surrounding the deep colony. It may happen that, after 24 hours incubation, a culture may present the gamma appearance; after 48 hours the colonies may be surrounded by slight greenish discoloration; after refrigeration of the plate overnight, typical zones of alpha hemolysis may develop. This trend of events is rather characteristic of *Strep. faecalis*.

¹ Enterococci have the Lancefield group D polysaccharide.

² A Quellung reaction of the pneumococcus capsule occurs with specific antiserum and offers a rapid method of recognizing a pneumococcus. (See par. 304.)

and female genital tracts. In puerperal sepsis due to these streptococci, infection appears to be autogenous.

c. **DIAGNOSTIC CRITERIA.** The group may be defined as strictly anaerobic gram-positive cocci in chains or clusters, usually producing a foul odor and gas. From the viewpoint of pathogenicity the differentiation of species is of no known significance.

340. *Diplococcus Pneumoniae* (*Pneumococcus*)

a. **CHARACTERISTICS.** In direct smears from infected material, these oval cocci usually appear in pairs, the long axis of the cell coinciding with that of the pair. In fluid media, short chains are frequently present, and with some strains this tendency is marked. Capsules are seen, especially in infected material, and they appear as clear, unstained halos around the organism. The gram-positive staining property is frequently lost early in the life of the culture owing to partial autolysis of the organism. Autolysis is accelerated by bile, giving the basis for the bile solubility test. (See par. 292.) Growth is best on blood-enriched media (either blood agar plates or blood broth). Twenty-four-hour colonies are about 1 mm in diameter and usually show central flattening, especially when crowded. There is a narrow zone of greenish discoloration of the blood (alpha hemolysis) around the colony. Inulin is fermented. Growth is aerobic and facultatively anaerobic. The organisms are susceptible to heat and the usual chemical disinfectants. There are more than 31 serological types of pneumococci, having different capsular polysaccharides. The common types are designated by the numerals 1 to 33, less types 26 and 30, which are identical with types 6 and 15, respectively.

b. **HOST RELATION.** The pneumococcus is the commonest cause of bacterial pneumonia. Even though it is frequently present in the normal upper respiratory tract, there is evidence that infection is not ordinarily autogenous. Invasion of the blood stream occurs in about one-fourth of the cases, with a consequent increase in mortality. The pneumococcus may also be responsible for otitis media, conjunctivitis, meningitis, peritonitis, and a variety of other infections.

c. **DIAGNOSTIC CRITERIA.** Morphology is of little aid in distinguishing pneumococci from streptococci. Identification is based on serological reactions, principally the Neufeld reaction (par. 304), on bile solubility, and on inulin fermentation. The first is directly applicable to the specimen, usually sputum, and is the most rapid. If positive, it is ordinarily sufficient to establish the bacteriologic diagnosis. A blood agar plate should also be inoculated to determine the presence of other gram-positive organisms,

including nontypeable pneumococci. Inulin fermentation is usually reliable but is the least constant of the three criteria. A mixture of types of pneumococci is found in some specimens.

d. **SPECIAL ISOLATION METHODS.** Specimens of sputum containing too few pneumococci to be satisfactorily typed by the Neufeld method may be "enriched" by mouse inoculation or the Avery method.

(1) *Mouse inoculation.*

- (a) Wash a selected portion of the sputum in sterile physiological salt solution in Petri dish and emulsify the washed sputum by means of a syringe.
- (b) Inoculate a mouse intraperitoneally with 0.5 to 1.0 ml of the emulsified sputum.
- (c) At intervals, after 6 hours, aspirate a little peritoneal fluid by puncturing the abdominal wall of the mouse with a fine glass capillary tube. Smear and examine the fluid for pneumococci.
- (d) When the pneumococci are found, kill the mouse and wash out the peritoneal cavity with a small amount of salt solution.
- (e) Use this suspension for Neufeld typing, diluting it if necessary.
- (f) Pure cultures of the pneumococcus may often be isolated from the heart's blood or the peritoneal fluid of the mouse provided aseptic technic is employed. Such cultures may also be used for typing.

- (2) *Avery method ("artificial mouse").* If mice are not available, substitute several tubes of broth (par. 272i) to which 0.25 ml (or 5 drops) of sterile defibrinated blood or 0.5 ml of sterile serum have been added. Inoculate with graded quantities of the sputum and examine at intervals after 4 to 6 hours' incubation.

341. Gram-negative Cocci—*Neisseria* *Meningitidis* (*Meningococcus*)

a. **CHARACTERISTICS.** The meningococcus is a spherical organism usually seen in pairs with the adjacent sides flattened. In infected spinal fluid it is usually found inside the polymorphonuclear leukocytes. With most strains no growth, or very scanty growth, takes place on ordinary nutrient agar. Serum or blood and a moist environment are required for multiplication. Better growth is seen on heated-blood (chocolate) agar. An atmosphere containing an increased amount of carbon dioxide is preferred for primary isolation. After 24 hours at 35° C. the colony is about 1 mm

in diameter, bluish-gray, and translucent. Growth does not occur under anaerobic conditions, or below 30° C. (For carbohydrate fermentations, see table XXIX.) The oxidase reaction is positive. These organisms are exceptionally sensitive to heat, chemical disinfectants, and drying. Cultures undergo rapid autolysis and die in a few days when kept at room temperature. Meningococci are currently separated into three distinct serological groups by agglutination, groups I, II, and *II alpha*. Group *II alpha* is closely related to group II and contains part of the antigenic structure of the ordinary group II and an additional antigen. The group *II alpha* organism, as compared with the group II strain, is characterized by being pathogenic to mice and having a capsule.

b. HOST RELATION. Cerebrospinal meningitis in epidemic form is due generally to the group I, rarely the group *II alpha*, meningococcus. A considerable number of persons normally carry these organisms in the nasopharynx, but many of these strains belong to group II, the members of which are relatively nonpathogenic. During an epidemic, the number of group I carriers increases markedly, and a certain number of these carriers may develop meningitis under suitable conditions. The organism may be isolated from the spinal fluid. In a certain proportion of advanced cases it may be cultivated from the blood stream, petechial skin lesions, and sometimes from the nasopharynx early in the disease. In the less common septicemic type of meningococcal infection, the organisms are found in the blood stream and appear only late, if at all, in the spinal fluid. These infections are often rapidly fatal.

c. DIAGNOSTIC CRITERIA. The normal spinal fluid is water-clear and colorless, and in meningitis it is more or less turbid. The color and turbidity, and the presence of blood and any clotting should be noted. Blood, if fresh, may have come from the spinal puncture and makes examination of the fluid difficult. An immediate presumptive diagnosis of meningococcal meningitis may be made by direct study of cerebrospinal fluid. Centrifuge the fluid, prepare spreads of the sediment on glass slides, fix, and stain by the Gram method. Examine for typical gram-negative, coffee-bean-shaped, *intracellular* diplococci. If present, they should be considered as meningococci and tentatively reported as such, to be confirmed by culture and agglutination tests. The presence of other organisms is also reported. Make total and differential counts, comparable to the counting of blood cells. The relative number of polymorphonuclear leukocytes is usually enormously increased in cerebrospinal meningitis. Plant several loopfuls of sediment on a warm chocolate agar plate. Inoculate a tube of warm tryptose phosphate broth or of meningococcus semisolid agar with 1 ml of spinal fluid. An alternate method, especially

valuable for use in small laboratories is to place 2 ml of the spinal fluid on the surface of a chocolate glucose agar slant that is plugged with a cork; thorough flaming of the mouth of the tube, firing the cork, and immediate insertion of the cork into the mouth of the tube will give sufficient carbon dioxide for the promotion of growth. Incubate cultures at 35° C. for 18 to 24 hours, in an atmosphere preferably containing an increased amount of carbon dioxide and observe for typical gram-negative, coffee-bean-shaped diplococci. Cultures are generally pure; if mixed, pure growth may be obtained by subcultures on solid media (as for the gonococcus). Pure cultures are used for fermentation tests to rule out *N. gonorrhoeae* and for tube agglutination tests.

d. SPECIAL METHODS.

- (1) *Culture of blood.* Blood, as well as spinal fluid, should be cultured from all suspected cases. Culture 3 to 5 ml of blood in a flask containing 100 ml of special purpose broth (par. 272i); the media should contain 0.0002 percent para-aminobenzoic acid if the patient is known or is suspected of having been treated with sulfa compounds. In cases of suspected meningococcemia showing petechiae, stained smears and cultures of these lesions may accelerate diagnosis.
- (2) *Culture of nasopharynx.* This is done for the detection of carriers. The nasopharynx of a convalescent or of a potential carrier is touched with a sterile cotton swab, preferably on a slightly bent wire (see par. 223), and the inoculum is spread diffusely over warm blood agar or chocolate agar plates. After incubation at 37° C., suspicious colonies are fished to a warm chocolate agar plate for confirmation of identity; the oxidase test should be used, and oxidase-positive colonies selected for further study. (See par. 291.)
- (3) *Agglutination tests of pure cultures.* A presumptive slide agglutination may hasten the procedure and eliminate atypical organisms. A macroscopic tube agglutination test with polyvalent antimeningococcus serum is used for final proof of identity. Occasionally group determination is indicated. Only polyvalent serum is issued to small hospital laboratories. When further study is indicated, or when directed by higher authority, subcultures on cork-stoppered chocolate glucose agar slants should be forwarded to the army area or other laboratory. The techniques of the agglutination tests for grouping meningococci are given in paragraph 305. Since most of the saprophytic *Neisseria* are salt or serum sensitive, it is

necessary in all agglutination tests for meningococci to run controls using normal horse serum (diluted 1:10) and saline, in order to rule out nonspecific clumping.

- (4) *Fermentation reactions.* With material from a pure culture, inoculate tubes of semisolid fermentation medium (see par. 271e(1)) and the four pivotal sugars. (See table XXIX.) Incubate cultures at 35° C. for 3 to 7 days. Such cultures, as well as those for the identification of the gonococcus, should *not* be incubated in an atmosphere containing increased carbon dioxide, which affects the indicator.

342. *Neisseria Gonorrhoeae* (*Gonococcus*)

a. CHARACTERISTICS. The gonococcus is similar to the meningococcus in many respects. Gram-negative paired cocci with adjacent sides flattened and fairly regular in size and shape, appear in direct smears of infected material, *usually inside polymorphonuclear leukocytes*. Growth is best on moist chocolate agar in an atmosphere containing increased carbon dioxide. In 24 hours the colony is about 1 mm in diameter, glistening, translucent, and grayish-white. In culture, the cocci show variation in size and shape. No growth occurs on ordinary nutrient media, at room temperature, or in the absence of oxygen. As with all the *Neisseria*, the oxidase reaction is positive. (For the fermentation reactions that help to distinguish the meningococcus from the gonococcus see table XXIX.) Gonococci are highly susceptible to inimical agencies. When dried, the cocci die in 2 hours; moist heat at 55° C. kills them in 5 minutes; they are quickly killed by a 0.025 percent solution of silver nitrate; cultures kept at room temperatures die in a few days, but at 37° C. they may survive several weeks.

b. HOST RELATION. The gonococcus is a strict parasite, the causative agent of gonorrhoea. In the male, the primary infection appears in the anterior urethra; extension often occurs from this site to the posterior urethra and prostate gland, where a chronic infection may be established. In the female, the urethra and sometimes the rectum are involved, with extension to the uterine cervix and fallopian tubes, where a chronic infection is often established. The organisms may gain access to the blood stream and localize in the joints, causing arthritis. Very rarely a gonorrhoeal endocarditis is seen. New born infants, due to passage through an infected birth canal, may acquire a gonorrhoeal conjunctivitis (ophthalmia neonatorum), which may result in blindness. Adults are also susceptible to eye infections with the gonococcus. In young

Table XXIX. Differentiation of *Neisseria*

Species	Fermentation tests				22° C. growth	Agglutination with meningococci serum	Special colony feature
	Dextrose	Maltose	Levulose	Sucrose			
<i>N. gonorrhoeae</i>	A	—	—	—	—	—	Small, round, convex.
<i>N. intracellularis</i> ..	A	A	—	—	—	+	Small, round, bluish-gray.
<i>N. catarrhalis</i>	—	—	—	—	+	—	Large, grayish-white.
<i>N. sicca</i>	A	A	A	A	+	—	Large, wrinkled, impossible to emulsify.
<i>N. perflava</i>	A	A	A	A	+	—	Greenish-yellow, adherent to medium.
<i>N. flava</i>	A	A	A	—	—	—	Yellow.
<i>N. subflava</i>	A	A	—	—	±	—	Greenish-yellow, adherent to medium.
<i>N. flavescens</i>	—	—	—	—	?	—	Golden yellow.

A indicates formation of acid.

girls gonorrhoeal vulvovaginitis occasionally occurs, being especially prevalent in institutions.

c. DIAGNOSTIC CRITERIA. A presumptive diagnosis may be made on microscopic examination; cultural confirmation should be done when necessary.

d. SPECIAL METHODS.

- (1) *Microscopy*. Make direct spreads of the infected urethral, cervical, or conjunctival discharges on glass slides, fix with heat, and stain by the Gram method. Examine the stained preparation for gram-negative, coffee-bean-shaped, intracellular (or extracellular) diplococci. Report whether the diplococci are intra- or extra-cellular, or both. Also report any other bacterial forms present, noting for each whether gram-negative or gram-positive and whether coccus or bacillus; also the relative numbers and kinds of tissue cells present.
- (2) *Cultivation*. Since ordinary cultural methods, especially in chronic urethral or cervical infections, reveal only the



Figure 27. Gonorrhoeal pus. (Film from urethra showing intracellular and extracellular *N. gonorrhoeae*.)

secondary organisms, a special culture program is needed for growing *N. gonorrhoeae*. The cultural demonstration of the gonococcus is superior to direct spread examinations in cases of chronic gonorrhoea in both sexes and in all cases in the female, especially when material for examination is taken from the cervix. The cultivation of the gonococcus when mixed with freer-growing micro-organisms requires observance of the following special procedures:

- (a) Take specimens of representative material and apply directly to plate media.
 - (b) Use a medium which will readily support growth of the gonococcus such as fresh, warm, moist chocolate agar or preferably, one of the media given in paragraph 272*w*.
 - (c) Grow in an atmosphere containing an increased amount of carbon dioxide (see par. 264) at 35° C. for 48 hours. If the recommended media are used, growth may be observed in 24 hours.
 - (d) Identify the gonococcus group by colony form and oxidase reaction.
 - (e) Confirm the identification by carbohydrate fermentation tests.
- (3) *Specimen taking and inoculation (methods listed in order of preference).*
- (a) Touch the platinum loop to a drop of pus, to the urethra, or the cleansed cervical os, and immediately streak broadly over a warm culture plate at the bedside or clinic chair.
 - (b) Similarly contaminate a sterile swab with the suspected material at the bedside or clinic chair and immediately place in a tube containing 1 ml of nutrient broth for prompt transmission to laboratory and inoculation of warm culture plate (broad spread of 0.1 ml of this broth).
 - (c) Inoculate the sediment from a centrifuged urine specimen on a warm culture plate. Preferably use the first portion of an early morning specimen.
 - (d) For delayed inoculation (up to 8 hours), store the above swab-broth tube in an ice box until the inoculation is made. If horse blood is available, swabs may be placed in tubes containing about 1 ml of sterile defibrinated blood, which permits survival of the gonococci for one or several days at ordinarily encountered

room temperatures. Delayed inoculation should be avoided if at all possible.

(4) *Examination of culture.* Observe for two features:

(a) The colonies are convex, slightly opaque, and 1 to 3 mm in diameter, with undulated margins. Their slight opacity and characteristic undulated margins serve to differentiate them from colonies of streptococci and diphtheroids.

(b) Positive oxidase reaction.

343. Nonpathogenic *Neisseria*

These organisms appear in the normal nasopharynx, often in large numbers. Most of them grow on plain nutrient agar and at 20° C. which helps to distinguish them from the pathogenic *Neisseria*. The colonies are rather small and may resemble those of the meningococcus. Some species produce a yellow pigment. There is a tendency to agglutinate spontaneously in normal saline solution or in normal horse serum. One of the commonest species, *N. catarrhalis*, fails to ferment carbohydrates. Table XXIX gives the distinguishing characteristics for this and other species. The growth requirements, fermentation reactions, and serological behavior serve to differentiate them from the meningococcus and gonococcus.

344. Tribe MIMAE

Certain organisms which appear as *Neisseria* on agar media and are frequently obtained from the genito-urinary passages, produce rod forms in broth. These have been placed in the tribe *Mimae*. The name is derived from the Latin word "mimus" meaning impersonator. They show resemblance also to *Klebsiella* and *Paracolobactrum*.

ENTERIC GRAM-NEGATIVE BACILLI— COLI-AEROGENES-FRIEDLÄNDER GROUP

345. Coli-Aerogenes-Friedlander Group (Tribe Eschericheae)

This group consists of three genera: *Escherichia coli* (formerly called *B. coli*), *Aerobacter aerogenes* and *Klebsiella*. The group consists of motile or nonmotile rods, commonly occurring in the intestinal canal of man and other animals or in the respiratory tract of man (genus *Klebsiella*), and widely distributed in nature. All ferment dextrose and lactose with the formation of acid and gas, with the exception of certain strains of *Klebsiella*, which do

not ferment lactose. They do not liquefy gelatin except for one species (*Aerobacter cloacae*), which liquefies it slowly. Members of this group are separated into three genera on the basis of the results of the methyl red test, the Voges-Proskauer test, and their ability to utilize citric acid as sole source of carbon. (See table XXXI.) Most organisms belonging to this group may be readily distinguished from nonlactose-fermenting pathogenic species by the typical colonies they form on EMB agar and other lactose-containing differential plate media, and the type of reaction on Kligler's sugar medium (acid slant, acid and gas in butt). In the Department of Bacteriology, Army Medical Service Graduate School, all non-pigmented, gram-negative rods which are biochemically active over a wide range of types of carbohydrates within 48 hours, but are not typical *Escherichia* or *Aerobacter* are termed *Atypical Escherichia* if VP negative and MR positive or *Atypical Escherichia* if VP positive and MR negative. Although the *Klebsiella* are poorly defined, the name is retained for the present for those organisms so designated by usage.

346. *Escherichia Coli* (*Bacterium Coli*, *Colon Bacillus*)

a. CHARACTERISTICS. The initial step in the recognition of the colon bacillus rests upon its rapid fermentation of lactose with the production of acid and gas. This is the basis for many differential media that are used in plating out feces for the purpose of separating the normally present colon bacilli from the pathogens, which ferment lactose slowly or not at all. Table XXX gives a description of the colony characteristics on some of these media. *E. Coli* is methyl red positive, produces indol, fails to utilize sodium citrate as a source of carbon, and fails to produce acetyl-methyl-carbinol from dextrose (Voges-Proskauer negative). Some strains are hemolytic. Motility is variable, but the majority of strains are motile.

b. HOST RELATION. Although essentially nonpathogenic, the colon bacillus may cause chronic infection of the urinary tract, and may be responsible for food poisoning. It is almost invariably present in the normal colon and lower portion of the ileum. As a result it is involved in mixed infections of the peritoneal cavity following intestinal perforation. It may occur in mixed wound infections. Its presence in water is used as an indication of fecal contamination.

c. DIAGNOSTIC CRITERIA. The demonstration of characteristic gram-negative nonspore-forming bacilli growing well on ordinary nutrient media with opaque nonmucoid colonies, and producing acid and gas from lactose in 24 hours establishes the identity of

Table XXX. Colony and Growth Characteristic of the Gram-Negative Intestinal Bacilli on Differential Media

Medium	Eosin-methylene blue agar plate	Deoxycholate-citrate agar and SS (Salmonella-Shigella) agar plates	Kligler's Iron Agar (with Phenol red indicator alkaline is red; acid, yellowish) *		H ₂ S
			Slant	Butt	
Mechanism	Coli-aerogenes group ferment lactose and grow into large, opaque colonies; also absorb dye to give color to colony. Nonlactose-fermenting pathogenic species develop as small, colorless, translucent colonies.	Lactose-fermenting organisms either are completely inhibited or produce reddish colonies. Nonlactose-fermenting organisms grow into small, clear, colorless, translucent colonies.	The small amount of acid produced from dextrose (0.1 percent) is diffused, leaving alkaline slant. The larger amount of acid from lactose (1 percent) gives acid slant.	Organisms producing acid from either dextrose or lactose give acid butt, with or without gas (bubbles in medium).	See par. 274e
<i>Escherichia</i>	Large colonies with large, dark (almost black) centers, and with greenish metallic sheen.	Much inhibited; if any growth, it is pink and opaque, opacity spreading to surrounding medium.	Acid	Acid and gas (marked).	+ and -
<i>Aerobacter</i> and <i>Klebsiella</i> .	Large pinkish mucoid colonies with small, dark brown or black centers; rarely show metallic sheen.	Same as <i>Escherichia</i> .	Acid, returning to neutral or alkaline after several days.	Acid and gas (marked).	-

<i>Salmonella</i>	Translucent, colorless, or pinkish colonies, later may have bluish tint.	Large translucent colonies, domed, shiny, smooth, and colorless.	Alkaline.....	Acid and gas (moderate). <i>S. typhi</i> and <i>S. gallinarum</i> are anaerogenic. Acid.....	+ (few -)
<i>Shigella</i>	Small, translucent, colorless colonies	Same as <i>Salmonella</i> colonies, except smaller. After 24 hours, colonies of <i>Sh. sonnei</i> may be red.	Alkaline, <i>Sh. sonnei</i> may produce acid after several days.	Acid.....	-
<i>Proteus</i>	Translucent, colorless, spreading colonies.	Same as <i>Salmonella</i> , spreading inhibited.	Alkaline.....	Acid and gas.....	+ and -
<i>Paracolon</i> s.....	Same as <i>Salmonella</i>	Same as <i>Salmonella</i> . After 24 hours, may be red.	Alkaline. May produce acid after several days.	Acid and gas or acid only.	+ and -
<i>Pseudomonas</i>	Same as <i>Shigella</i>	Same as <i>Shigella</i>	Alkaline; usually purplish.	Unchanged or acid.....	-
<i>Alcaligenes</i>	Same as <i>Shigella</i>	Same as <i>Shigella</i>	Alkaline.....	Unchanged.....	-

* Triple sugar iron agar gives the same reactions except that many paracolons will produce an acid slant due to sucrose fermentation.

E. coli for all practical purposes. To distinguish it from *E. freundii*, sometimes referred to as the intermediate type, and from *Aerobacter aerogenes*, see table XXXI.

Table XXXI. Differentiation of the Coli-Aerogenes-Friedländer Group

Species	Methyl- red test	Voges- Proskauer reaction	Indol Produc- tion	Citrate Utiliza- tion	Gelatin lique- faction	Hydrogen sulfide formation
<i>Escherichia coli</i>	+	—	+	—	—	—
<i>Escherichia freundii</i>	+	(—)	(+)	+	—	+
<i>Aerobacter aerogenes</i>	—	+	(—)	+	—	(—)
<i>Aerobacter cloacae</i>	—	+	—	+	+	(—)
<i>Klebsiella pneumoniae</i> ...	(+)	(—)	—	(+)	—	—

The reactions inclosed with parentheses are the usual ones; exceptions have been reported.

347. *Aerobacter Aerogenes*

a. CHARACTERISTICS. This organism resembles the colon bacillus in that it forms acid and gas from lactose, but differs from it in certain respects. Microscopically the organisms are shorter and thicker than *E. coli*, are nonmotile, and are often encapsulated. Gas production is more vigorous, indol is not formed, citrate is utilized, the Voges-Proskauer reaction is positive, and the methyl-red test negative. (See table XXXI.)

b. HOST RELATION. Organisms belonging to this species are normally found on grains and plants, and to a varying degree in the feces of man and animals. They are widely distributed in nature. They may cause low grade urinary tract infections; aside from this they are essentially nonpathogenic. Differentiation from *E. coli* is sometimes desired since in water supplies *A. aerogenes* may be of nonfecal origin.

c. DIAGNOSTIC CRITERIA. The demonstration of short, plump, gram-negative rods, usually encapsulated, growing well on ordinary nutrient agar with a raised white mucoid colony, and fermenting lactose with acid and gas, will usually be sufficient to establish identity. (See table XXXI for more precise identification.)

348. *Klebsilla Pneumoniae* (Friedländer's Bacillus, Bacillus Mucosus Capsulatus)

a. CHARACTERISTICS. Friedländer's bacillus is a short, thick, gram-negative, nonmotile rod, which, particularly in exudates, is heavily encapsulated. It grows well on ordinary nutrient agar forming semiopaque mucoid colonies 3 to 4 mm in diameter. Gelatin is not liquefied. Lactose may or may not be fermented with

acid and gas formation. (For other reactions see table XXXI.) Bromcresol purple or litmus milk often serves to differentiate *K. pneumoniae* and *A. aerogenes*, the latter producing acid and coagulation promptly, the former not. *K. pneumoniae* is similar to *A. aerogenes* in many respects and some strains are closely related serologically. Serologically, the strains have been divided into types A, B, C, and X (Julianelle).

b. HOST RELATION. *K. pneumoniae* may be found in the normal respiratory tract of man. It may cause lobar pneumonia with an associated septicemia. It may also appear as a secondary invader in wounds. Intraperitoneal inoculation of mice is usually fatal in 24 to 48 hours.

c. DIAGNOSTIC CRITERIA. Short, thick encapsulated, gram-negative nonmotile rods growing with a mucoid colony and isolated from the respiratory tract are usually termed *K. pneumoniae*, although these organisms are not clearly distinct from *Aerobacter aerogenes*. If typing antisera are available, *K. pneumoniae* may be typed by the capsule-swelling reaction. The majority of human pathogenic strains belong to type A.

349. *Salmonella* and *Shigella*. General Remarks

An outline is given which should aid in isolating and identifying organisms belonging to these groups of enteric pathogens.

a. OBTAIN SPECIMEN (see par 231).

- (1) Fresh stool and/or rectal swab.
- (2) Feces in buffered glycerine-saline solution.

b. INOCULATE PLATE MEDIA.

- (1) Inoculate appropriate plate media and enrichment broths (par. 272 a—eQ. Enrichment broths are most valuable after the acute phase of the disease is passed and in carrier studies.
- (2) Inoculate plate media from enrichment broth after 24 hour incubation.

c. INOCULATE IRON AGAR.

- (1) Kligler's iron agar (KIA) or triple sugar iron agar are inoculated with suspicious colonies (see table XXX).
- (2) After 24 hours, tubes are separated according to reactions which are given in the same table.

d. KIA CULTURES PRODUCING HYDROGEN SULFIDE, BUT SHOWING ALKALINE SLANT WITH ACID OR ACID AND GAS IN THE BUTT.

- (1) Inoculate rapid urease test medium (see par. 280). If the test is positive, the culture belongs to the *Proteus* group.
- (2) If rapid urease test is negative, test culture with *Salmonella* polyvalent antiserum.

(3) If the polyvalent serum test is negative, study motility and biochemical tests (*f*).

(4) If the polyvalent serum test is positive, identify as far as possible serologically. Perform motility and biochemical tests (*f*). This is important inasmuch as many paracolon organisms possess *Salmonella* antigens.

e. KIA CULTURES PRODUCING NO HYDROGEN SULFIDE, BUT SHOWING AN ALKALINE SLANT, WITH ACID OR ACID AND GAS IN BUTT.

(1) Test with *Shigella* grouping or *Salmonella* polyvalent antisera. (*Shigellae* generally fail to produce gas in KIA. Exceptions are indicated in tables XXX and XXXII).

(2) If serological tests are negative, study motility and biochemical tests (*f*).

(3) If serological tests are positive, identify as far as possible serologically. Perform motility and biochemical tests (*f*) bearing in mind that many paracolon organisms possess *Salmonella* or *Shigella* antigens.

f. BIOCHEMICAL AND MOTILITY TESTS.

(1) Carbohydrate fermentation tests. Inoculate broths containing dextrose, lactose, sucrose, mannitol, salicin and adonitol. Others e.g. sorbitol and maltose also may be inoculated if desired. (See par. 274a.)

(2) Urease test. Inoculate 24-hour urease test medium (par. 280b).

(3) Test for citrate utilization. Inoculate Simmons' citrate agar. (See par. 287.)

(4) MR-VP tests. Inoculate Clark and Lubs Medium. (See pars. 274c, 278, and 279).

(5) Indol test. (See par. 283.)

(6) Hydrogen sulfide test. (See par. 285.)

(7) Test for motility. (See par. 271e.)

(8) Test for gelatin liquefaction. (See par. 286.)

(9) Test for nitrate reduction. (See par. 289.)

g. TYPICAL SALMONELLA AND SHIGELLA BIOCHEMICAL REACTIONS. See table XXXII.

Caution: Although serological identification is extremely important in studying the *Salmonella* and *Shigella* groups, biochemical verification should not be neglected, particularly in view of the occurrence of *Salmonella* and *Shigella* antigens in other groups. Also, organisms failing to give typical reactions should not be passed-over without thought. All types of known pathogenic groups have not yet been discovered, and the possible pathogenic role of members of others groups must be considered. Likewise,

the isolation of a pathogen, particularly from enrichment broth, should not divert attention from other possibilities.

350. Genus *Salmonella*

a. CHARACTERISTICS. These are non-pigmented gram-negative rods which grow well on ordinary media, ferment dextrose (usually producing acid and gas), are usually motile, do not ferment lactose, sucrose or salicin, are urease negative, indol negative, VP negative and gelatin negative and usually ferment mannite and sorbitol. Accurate identification depends upon the serological analysis of somatic (O) and flagellar (H) antigenic components (see par. 306). It has become customary to indicate antigenic types as "species."

b. HOST RELATION. Members of this group are responsible for a variety of types of infection although paratyphoid fever and food-poisoning are most frequently associated with them. Accompanying the emphasis on antigenic characterization, the etiologic agent of typhoid fever is now generally placed with the *Salmonella*, although it does not produce gas in dextrose. Evidence for the pathogenicity of some *Salmonella* is lacking.

c. DIAGNOSTIC CRITERIA. Cultures showing the characteristics indicated above (see table XXXII) may be isolated from food, feces and vomitus of infected individuals in food poisoning outbreaks due to members of the *Salmonella* group. Organisms may be isolated from the stools of carrier food handlers, particularly if enrichment broth is inoculated. In cases of enteric fever, specimens of feces, urine, gallbladder drainage, and blood should be studied. Serum for the agglutination test is of interest after the seventh day of the disease. Organisms tentatively identified as belonging to this genus should be forwarded to an Army Area Medical Laboratory or other reference laboratory, accompanied by a brief clinical abstract of the case.

351. *Salmonella Typhi* (*Eberthella Typhosa*, Typhoid Bacillus)

a. CHARACTERISTICS. Except for failure to produce gas from sugars, this organism is culturally and antigenically typical of the *Salmonella*. It is placed in Group D since it contains somatic antigens IX and XII. (Table XXVI.) Freshly isolated stains also usually possess the additional Vi antigen which may mask agglutination with "O" serum. The flagellar antigen of *S. typhi* is monophasic. Instructions for detecting the antigens of *S. typhi* are contained in the instructions accompanying *Salmonella* typing kits. (See par. 306.)

b. HOST RELATION. The typhoid bacillus appears in the blood

stream during the first 7 to 10 days of the disease, gradually disappearing during the following 2 weeks. As the disease progresses, the organisms in the feces increase in number, reaching a maximum at about the third week. There is usually localization of the organisms in the gall bladder; if chronic infection of this organ results, a persistent and potentially dangerous carrier state is established. The urine often contains typhoid bacilli during the disease, and they may persist for months or years after recovery. Specific agglutinins appear in the patient's blood at about the fifth to tenth day, the titer gradually increasing. The presence of these antibodies is determined by the Widal test.

c. **DIAGNOSTIC CRITERIA.** It is necessary to demonstrate motile gram-negative rods with characteristics typical of the *Salmonella* (except for gas production) and containing the appropriate antigenic pattern indicated in paragraph 351a.

d. **SPECIAL METHODS.** The specimens to be examined culturally usually consist of blood, feces, urine, and bile of suspected cases of typhoid or paratyphoid fever and of bile, feces, and urine of carriers; also, serum from patients may be examined by the agglutination (Widal) test.

(1) *Culture.*

- (a) *Feces, urine, bile.* Spread the material (if solid feces, emulsify in broth or saline) over the dry surface of EMB agar, desoxycholate-citrate agar, or other special differential media in Petri dishes, in such a manner as to insure the growth of well isolated colonies. Also, inoculate the specimen into a tube of selenite-F broth or bile broth. Incubate 18 to 24 hours at 35° C. Study the plate cultures carefully. Select several well-isolated colonies of the type desired (table XXX), and from each inoculate a tube of Kligler's iron agar and a tube of nutrient broth. After 24 hours' incubation examine cultures for type reaction on Kligler's motility, and gram-staining properties. Identify any suspected pure culture, by inoculating various carbohydrate media and media for the other biochemical tests, and eventually set up macroscopic agglutination tests against known antisera of the suspected type. If at the end of 24 hours, plate cultures show no colonies of the type produced by pathogenic organisms, streak new differential plates from the enrichment medium and reincubate the old plates for an additional 24 hours before discarding as negative. If identification of the organism has not been determined, send transplants

to the nearest army area or other reference laboratory for further study.

- (b) *Blood*. Blood for culturing should be taken early in the disease, preferably during the first week. Obtain 10 to 15 ml of citrated or defibrinated blood (whole blood can be used for immediate inoculation of media at the bedside). Flask containing 100 ml of tryptose broth, 1 percent dextrose infusion broth, or semisolid medium with 5 to 10 ml of blood, make two agar pour plates containing 1 ml of blood each, and streak two or three loopfuls of blood on an EMB agar plate. Incubate the cultures at 37° C., and make transfers at intervals

Table XXXII.
Typical Confirmatory Reactions of Salmonella and Shigella Organisms

	<i>Salmonella</i>	<i>Shigella</i>
Dextrose.....	Acid and gas ¹	Acid ²
Lactose.....	—	Variable ³
Sucrose.....	—	Variable ³
Mannitol.....	+ (usually)	See par. 352
Salicin.....	—	—
Adonitol.....	—	—
Sorbitol.....	+ (usually)	Variable
Urease.....	—	—
Citrate.....	Variable	—
Methyl red.....	+	+
Voges-Proskauer.....	—	—
Indol.....	—	Variable
H ₂ S.....	+ (usually) ⁴	— ⁵
Motility.....	+ ⁶	—
Gelatin.....	— (usually)	—
Nitrate.....	+	+

¹. *S. typhi* and *S. gallinarum* are anaerogenic. Rare anaerogenic strains of other types.

². Certain biochemical variants of *Shigella flexneri* 6 (Newcastle, Manchester) produce gas from fermentable substances.

³. When acid is produced, the reaction is delayed for several days.

⁴. *S. paratyphi* A and rare strains of *S. typhi* and other types do not produce H₂S.

⁵. The sensitive paper tests may show some H₂S production by some strains.

⁶. All motile rods may have nonmotile variants. *Salmonella gallinarum* and *S. pullorum* are nonmotile.

to blood agar or other suitable agar plates. If colonies develop, transfer to Kligler's and identify by the procedure outlined above.

(2) *Serological examination.*

- (a) *Identification of organism.* The macroscopic tube-agglutination test, as indicated above, can be used to confirm the identity of an organism isolated from cultures. Use a suspension of the suspected culture as antigen along with known antisera.

- (b) *Identification of antibodies.* After the first or second week, demonstrable antibodies develop in the blood of patients with enteric fever. These may be demonstrated by the macroscopic tube agglutination test, using the patient's serum and stock *S. typhi* (H) and *S. typhi* (O), *S. paratyphi*, and *S. paratyphi* (B) antigens. This is frequently but incorrectly referred to as a Widal test.
- (3) *Bacteriophage typing.* Typhoid bacilli may be separated for epidemiological purposes, into numerous specific types based on susceptibility to stable strains of bacteriophage. The scarcity of the reagents and the necessity for considerable training in performing the test require that cultures to be typed be forwarded to the Army Medical Service Graduate School.

352. Genus *Shigella* (Dysentery Bacilli)

a. **CHARACTERISTICS.** The *Shigella* group contains non-pigmented, non-motile, aerobic, generally anaerogenic, gram-negative, non-sporulating rods which grow well on ordinary media, ferment some carbohydrates, but do not acidify salicin, produce no acetyl-methyl-carbinol, are citrate negative and urease negative. Some species ferment lactose slowly (table XXXII).

b. **HABITAT.** Several species are pathogenic for man, causing bacillary dysentery. Other species may be found in the normal human intestinal tract. Several species are pathogenic to fowl and small animals.

c. **CLASSIFICATION.** As in the case of *Salmonella*, emphasis should be placed on antigenic factors in typing *Shigella* organisms. It is important, however, to make certain that characteristics other than the antigenic content of an organism being studied also conform with those of the group (table XXXII) since some paracolons share surface somatic antigens with *Shigellae*. The organisms presently included in the *Shigella* group are indicated in Table XXXIII. The terminology recommended by the Nomenclature Commission of the International Association of Microbiologists is used. It will be noted that *B. madampensis*, *ceylonensis*, *alkalescens*, and *dispar II* are excluded from the *Shigella* group. These organisms are frequently found in normal feces and are of doubtful pathogenicity. *B. alkalescens* is occasionally associated with dysentery and urinary tract infections. *Shigella sonnei* and *Sh. flexneri* 2 (paradysenteriae W) are common causes of dysentery.

353. Mannitol—Negative Types

(Group A, table XXXIII.)

a. The organisms in this group fail to ferment mannitol. They include *Sh. dysenteriae* Type 1 (Shiga-Kruse bacillus), Type 2 (Schmitz bacillus) and the Large-Sachs organisms (Types 3 through 7).

b. One subtype (4c) of *Shigella flexneri* 4 (Group B) also fails to ferment mannitol, but its antigenic and other characteristics require that it be placed in Group B.

354. Mannitol—Positive Types

(Groups B, C, and D, table XXXIII.)

a. All the types of *Shigella* other than those given in paragraph 353 ferment mannite. Members of Group B possess common antigens. They are given the name *Shigella flexneri*. Types 1 and 2 and 6 have been commonly encountered. Organisms formerly designated as Types X and Y are considered by many to be strains which have lost the type antigens of other members of Group B.

b. Group C includes those strains isolated by Boyd whose antigens do not conform with those of Group B. The biochemical reactions resemble those of group B.

c. Group D contains only the commonly found *Shigella sonnei*. Acid is formed in lactose and sucrose as well as mannite, but usually only after prolonged incubation. This type exists in two "phases" I and II. Phase II is often termed "rough," but this terminology is inaccurate and confusing, for there also exists a true rough variant. The variants should be designated as form I, form II and R (rough). The term "phase" should be reserved for variation in the *flagellar* antigens of enteric organisms.

d. *B. ceylonensis* (dispar), *B. alkalescens*, and *B. madampensis* possess heat labile "L" antigens common to the coliform group. Their pathogenicity, other than that as shown by *E. coli* under certain conditions, is questionable. The "Wakefield" bacillus hydrolyzes urea and is a *Proteus*.

355. Special Methods

a. MICROSCOPIC EXAMINATION. In bacillary dysentery, an early presumptive diagnosis can usually be made by direct microscopic examination of fresh fecal discharges:

- (1) Select portions of a very fresh specimen containing bits of mucus, bloody feces, or shreds of the exudate. Prepare thin films and stain with Wrights' or Giemsa's

Table XXXIII. Types of *Shigella*

Group A (Mannite not fermented)		Type	Old designation and remarks
<i>Shigella dysenteriae</i>		1	<i>S. shigae</i> , Shiga-kruse bacillus, etc.
<i>Shigella dysenteriae</i>		2	<i>S. schmitzii</i> , <i>S. ambigua</i>
<i>Shigella dysenteriae</i>		3	Q 771
<i>Shigella dysenteriae</i>		4	Q1167
<i>Shigella dysenteriae</i>		5	Q1030
<i>Shigella dysenteriae</i>		6	Q 454
<i>Shigella dysenteriae</i>		7	Q 902
			Large-Sachs group
Group B	Type	Subtype	Old designation and remarks
<i>Shigella flexneri</i>	1	1a	V
		1b	VZ
<i>Shigella flexneri</i>	2	2a	W
		2b	WX
<i>Shigella flexneri</i>	3		Z
<i>Shigella flexneri</i>	4	4a	103 (Boyd).
		4b	Also possesses group antigen present in 1b. and 3.
		4c	Mannite (-). <i>Sh. rabaulensis</i> .
<i>Shigella flexneri</i>	5		P. 119 (Boyd).
<i>Shigella flexneri</i>	6		88 (Boyd) Manchester-Newcastle bacillus.
<i>Shigella flexneri</i>	Variant		
	{ X type }		X
	{ Y type }		Y
			(Andrews and Inman).
Group C		Type	Old designation and remarks
<i>Shigella boydii</i>		1	170
<i>Shigella boydii</i>		2	P288
<i>Shigella boydii</i>		3	D1
<i>Shigella boydii</i>		4	P274
<i>Shigella boydii</i>		5	P143
<i>Shigella boydii</i>		6	D19
<i>Shigella boydii</i>		7	"Lavington," <i>S. elousae</i> .
			(Boyd).
Group D			Old designation and remarks
<i>Shigella sonnei</i>			Sonne-Duval bacillus.
			<i>S. ceylonensis</i> A.

Organisms excluded from the group are the following species, among others:

Ceylonensis B (dispar), *alkalescens* I, II (tiete), *madampensis*, *wakefield* (= *Proteus*).

stain. In addition, make cover glass preparations, both unstained and stained with Loeffler's methylene blue or a 1 percent aqueous solution of brilliant cresyl blue, in order to study the cells present.

- (2) If the disease is the bacillary type of dysentery, microscopic examination will show blood in varying amounts, but usually abundant early in the disease. Polymorphonuclear neutrophils form about 90 percent of the exudate; many of these show nuclear degeneration (ringing), and the cytoplasm frequently contains fat. Endothelial macrophages, which are present in varying numbers, are actively phagocytic and frequently contain engulfed bacteria, erythrocytes, and leukocytes; these undergo degeneration and form "ghost cells." Plasma cells are present and are more abundant early in the disease. The bacterial content is scanty.
- (3) The characteristic findings in stools from cases of amebic dysentery are given in chapter 13.

b. CULTURAL EXAMINATION. Dysentery bacilli may be isolated from the feces or rectal swabs of patients and carriers by the methods indicated above in the section covering the typhoid bacillus. Both EMB agar plates and either desoxycholate-citrate or SS agar plates should be inoculated routinely. The latter two media are especially favorable for the isolation of *Shigella*. The blood stream is not invaded in bacillary dysentery, hence blood cultures are not indicated. Final identification of the organisms isolated must be based upon complete biochemical and serological study.

c. SEROLOGICAL IDENTIFICATION.

- (1) The members of the genus *Shigella* make up a group of species and types having a complex serological interrelationship. Some of the species such as *Sh. dysenteriae* 1 and 2 and *Sh. sonnei* are relatively homogeneous serologically. Other species contain one or more antigenic factors common to other species, or consist of several races or strains that show widely variable antigenic relationships. The types included in *Sh. flexneri* show the greatest complexity and present the greatest difficulty in identification because of close immunological similarities. The original division of the group by Andrewes and Inman into five races (V, W, X, Y, and Z) has been modified and extended by Boyd and others. The V, W, and Z types have been retained and additional members added; at present six serological types are recognized. (See table XXXIII.) The validity of types X and Y has been questioned. They probably represent degraded laboratory variants, and are extremely uncommon if they occur at all in active infections.
- (2) Organisms suspected of belonging to the genus *Shigella*

can be rapidly and accurately identified in most cases by means of a slide agglutination technic using polyvalent and monovalent absorbed sera. Simplified kits containing polyvalent or grouping sera are available. Kits containing monovalent absorbed sera for the members of each group are available only to certain few key laboratories. Cultures identified only as far as available facilities permit, should be forwarded to a higher reference laboratory.

356. *Pseudomonas Aeruginosa* (*Bacillus Pyocyaneus*, *Bacillus* of Blue-green Pus)

a. CHARACTERISTICS. This is a small, slender, motile, gram-negative rod, growing readily on ordinary nutrient agar. The colony is rather large, spreading and translucent with a yellowish-green color. The surrounding medium is colored green as a result of the development of a soluble blue-green pigment. Growth is aerobic and facultatively anaerobic, but the pigment develops only in the presence of oxygen. It is usually more intense if grown on plain agar at 30° C. or at room temperature. Spores are not formed.

b. HOST RELATION. *Ps. aeruginosa* is essentially nonpathogenic, but often appears as a secondary invader, particularly in wound infections, where it imparts a characteristic blue-green color to the pus.

c. DIAGNOSTIC CRITERIA. The demonstration of characteristic motile, gram-negative rods that form a soluble blue-green pigment after 1 to 4 days incubation is sufficient to identify an organism as *Ps. aeruginosa*.

357. Genus *Proteus*

a. CHARACTERISTICS. *Proteus* organisms are non-pigmented gram-negative rods which grow well on ordinary media and which ferment dextrose, but not lactose, and whose chief distinguishing feature is the ability to rapidly produce urease which splits urea to form ammonia. The release of ammonia is indicated in urea medium by the change to alkalinity (reddening of the indicator). They are actively motile, with peritrichous flagella, and grow readily on ordinary media. They show a tendency to "swarm" (that is, to cover the entire surface of a plate with a transparent film of growth), particularly if the medium is moist. Freshly isolated strains are actively proteolytic, liquefying gelatin rapidly. Dextrose and generally sucrose and other sugars are fermented with the production of acid and a small amount of gas,

lactose is never fermented. (See table XXXII.) In mixed culture material, the swarming of *Proteus* frequently interferes with the isolation of other organisms. Sometimes this can be prevented by pouring a thin layer of sterile agar over the inoculated plate or by incubating the plate anaerobically, assuming that the other organisms sought can develop under such conditions. An effective method of preventing the swarming of *Proteus*, without inhibition of other organisms, is to add to the plating agar, with or without blood, about 0.25 ml of a 5 percent aqueous solution of chloral hydrate. Chloral hydrate renders the bacilli temporarily nonmotile. Nonmotile or O variants do not swarm. *P. morganii* differs from the other species of the genus in several respects: no sugars are fermented except the hexoses (dextrose, etc.) and rarely xylose and sucrose; gelatin is not liquefied; no "swarming" occurs except on soft (1 percent) agar incubated at room temperature.

b. HOST RELATION. *Proteus* organisms are frequently a cause of cystitis or occur as secondary invaders in wounds. Their presence favors the development of anaerobes. They are commonly present in feces. One strain of this organism is of importance since it agglutinates in the serum of patients having typhus fever (Weil-Felix reaction), although it has no etiologic significance in that disease. This strain is a nonmotile O variant, designated *Proteus* OX19. Another strain, *Proteus* Kingsbury OXK, is used in the same way in the diagnosis of scrub typhus (tsutsugamushi fever). *Proteus* OX2 has been used in the differential diagnosis of Rocky Mountain spotted fever. *P. morganii* is of interest because of its occasional association with diarrheal disorders.

c. DIAGNOSTIC CRITERIA. A motile, nonchromogenic, gram-negative bacillus that produces characteristic swarming and liquefies gelatin may be provisionally classified as a member of the genus *Proteus*. Further identification is rarely required. A failure to swarm on ordinary agar or failure to liquefy gelatin promptly, however, does not necessarily exclude *Proteus*.

358. Genus *Paracolon* (Paracolon)

a. CHARACTERISTICS. These gram-negative rods include diverse intergrading forms between other genera of *Enterobacteriaceae*. Originally applied to late lactose-splitters, the name is now used for various atypical members of the family. Many are more closely related biochemically or antigenically to one or another of the better defined genera than to each other. Non-pigmented gram-negative rods which grow well on ordinary media and ferment dextrose, but whose characteristics do not conform with

those given in the amended descriptions of the other genera of *Enterobacteriaceae* are placed in this "waste-basket" group. (See also *Mimeae*, par. 344.) Although the paracolons have been grouped depending on production of indol and acetyl-methyl carbinol, methyl red test or growth in citrate medium (IMViC test),¹ organisms within IMViC groups may be entirely unrelated by other biochemical or by antigenic criteria. Some strains split urea, but more slowly and weakly than is characteristic of *Proteus*.

b. HOST RELATIONS. Many of these forms are non-pathogenic or may assume pathogenicity under condition described for *E. coli* or *Proteus*. They are frequently isolated from the stools of normal persons, particularly when concentration broth media are used. There is evidence, however, that some may play an etiologic role in diarrhea, food poisoning and low-grade enteritis.

359. *Vibrio Comma* (*V. Cholerae*, Cholera *Vibrio*, Comma *Bacillus*)

a. CHARACTERISTICS. The cholera vibrio causes Asiatic cholera, an intestinal infection in which tremendous quantities of fluid are lost through the wall of the bowel and by vomiting. As the name implies, these are moderately small, slightly curved rods with rounded ends, often resembling a comma. They occur singly or two or more may be attached end to end, giving an S or spiral appearance. They frequently are described as resembling Chinese characters. They are gram-negative and very actively motile by means of a single polar flagellum. Growth is abundant on ordinary nutrient medium; 24-hour colonies are 1 to 2 mm in diameter, smooth, gray, and translucent. In broth a fragile pellicle develops. Peptone water cultures give a positive "cholera-red" reaction due to concurrent production of indol and reduction of nitrates. This reaction is also given by two saprophytic species. Gelatin is liquefied. Several carbohydrates are fermented with acid only. *V. comma* and other vibrios are markedly aerobic; they grow best in the presence of abundant oxygen, giving a rapid growth on the surface of liquid media. Growth is almost nil under strictly anaerobic conditions. The cholera vibrio is capable of developing rapidly in a fairly alkaline (pH 8 to 8.4) medium, which is inhibitory to most other intestinal organisms. These last two facts are made use of in the isolation of the organism from feces and water. There are several classified, and probably many unclassified vibrios, that may be isolated from feces or

¹IMViC is a mnemonic indicating indol (I), methyl-red (M), Voges-Proskauer for acetyl-methyl-carbinol (V) and citrate tests (C).

water which can be differentiated on serological and biochemical characteristics. There has been a tendency in recent years to separate the cholera vibrios into several types, but Kauffman has shown that these are different "O" forms of one type. Thus, although they may possess antigens which are not found in the other "O" forms, the type antigen is present in all. The "El Tor" strains also possess the type antigen, but differ from true cholera vibrios in their capacity for hemolysis. True cholera vibrios are not hemolytic.

b. SPECIAL METHODS. *V. comma* may be isolated from the stools or intestinal contents of cases or carriers, and from contaminated water or foods; it can be identified by microscopic, cultural, and serological methods.

- (1) *Specimen collection.* The "rice water" stools of cases and the feces of carriers are collected without the addition of glycerol or other preservative. Surface water is collected in a sterile liter flask or other container.
- (2) *Microscopy.* A presumptive diagnosis of suspected cases, not of carriers, may be quickly made by examining stained spreads of flakes of mucus from the "rice water" stool. Stain by the Gram method and with dilute carbolfuchsin. If Gram-negative, comma-shaped organisms are present, examine a hanging-drop preparation. A presumptive positive report may be made if large numbers of typical, actively motile vibrios are found. This finding must then be confirmed by cultural examination and serological tests.
- (3) *Cultivation:*
 - (a) *Feces.* Specimens of feces from suspected cases or carriers should be planted, using two or more loopfuls of intestinal mucus or liquid feces, with the least possible delay and incubated at 37° C. As media, use several tubes of alkaline peptone water (pH 8 to 8.4) and alkaline nutrient agar (pH 8 to 8.4) or Dieu-donne's agar. (See par. 272f.)
 - (b) *Water.* Water under test is placed in 100 ml amounts in sterile flasks. To each flask is added 10 ml of 10 percent peptone water. After 6 to 12 hours incubation at 37° C., transfer a portion of the surface growth to one of the media mentioned above.
- (4) *Presumptive tests.*
 - (a) After 6 to 8 hours incubation of the broth at 35° to 37° C., a loopful from the surface is examined by Gram stain and hanging-drop preparation. Whether or not gram-negative motile vibrios are found, streak

several loopfuls from the surface of the broth on alkaline agar plates and incubate these. Desoxycholate agar also may be used (par. 272b).

- (b) The colonies of *V. Cholerae* are semi-transparent and look like droplets of dew. Most of the other organisms are inhibited and the ones that grow are generally opaque.
 - (c) If gram-negative motile vibrios are noted in the broth, the "cholera-red" test may be performed, although this test is not as specific for the true cholera vibrios as once supposed. Add a few drops of commercial sulfuric acid to a 24-hour peptone water culture; a resulting red color (positive "cholera-red" test) depends on the nitrore-indol reaction from the production of indol and the reduction of nitrate in the peptone.
- (5) *Confirmatory Tests.*
- (a) If growth on the alkaline nutrient agar plates is sufficiently heavy and pure, agglutination tests may be made from these colonies. Otherwise growth should be obtained by streaking an isolated colony on agar slants and incubating for 12 hours. A polyvalent "O" serum containing antibodies agglutinating the Inaba and Ogawa strains is available and should be used in accordance with the procedure for performing a tube agglutination test (par. 299). True cholera as well as El Tor vibrios should agglutinate in serum dilutions approximating the labeled titer. Heated (1 hour at 56° C.) or live suspensions of vibrios may be employed as antigens.
 - (b) Test organisms agglutinating as indicated in (a) for hemolytic activity. Five percent (in saline) sheep and goat cells are prepared and dispersed into serological tubes in 2 ml quantities. These are inoculated with several loopfuls of a young culture, incubated at 35° to 37° C. for a few hours and inspected at intervals for hemolysis. If no hemolysis is observed, the organism is considered to be *Vibrio Cholerae* (epidemic type, Inaba or Ogawa). Hemolysis characterizes the El Tor vibrio. Isolated organisms should be forwarded to reference laboratories for fermentation studies and typing.

360. Genus *Brucella*

a. CHARACTERISTICS. Three species of significance to man occur in this genus—*Br. melitensis*, *abortus*, and *suis*, natural patho-

gens of goats, cattle, and hogs, respectively. They are small, very short, sometimes almost coccoid, gram-negative, nonmotile bacilli. Freshly isolated strains of *Br. abortus* usually show no growth unless they are incubated in an atmosphere containing 5 to 10 percent carbon dioxide. *Br. melitensis*, although not requiring increased carbon dioxide, generally grows better in its presence. *Br. suis* not only does not require increased carbon dioxide but growth is not improved by its presence. Good growth may be obtained with Trypticase Soy Broth and agar (par. 272i and j), although growth on all media is slow on first isolation. After 48 hours the colonies are 1 to 2 mm in diameter, grayish, and translucent. Agar cultures turn brownish after several days. No carbohydrates are fermented. In addition to differences in carbon dioxide requirements, the three species may be differentiated on the basis of susceptibility to various dyes and by hydrogen sulfide production. (See table XXXIV.) Tests show almost complete cross-agglutination among the three species.

Table XXXIV. Differentiation of *brucella*

Species	Increased atmospheric carbon dioxide required for primary isolation	Duration of hydrogen sulfide formation (days)	Growth on media containing—	
			Thionin	Basic fuchsin
<i>Br. melitensis</i>	—	±1	+	+
<i>Br. abortus</i>	+	2	—	+
<i>Br. suis</i>	—	4	+	—

b. HOST RELATION. *Br. melitensis*, *suis*, and *abortus*, listed in order of their virulence for man, are responsible for undulant fever and other forms of brucellosis. The route of infection may be cutaneous, conjunctival, or oral—the first two by contact with infected animals or animal products, the last by ingestion of raw milk or milk products from infected cattle or goats. The nature of the disease is extremely variable and chronic. The average duration is 3 months. The infection is generalized with localization in the lymphatic system. Early in the disease the organism may be cultivated from the blood stream, most often in cases due to *Br. melitensis*. After 2 to 3 weeks, the likelihood of isolation from the blood is not great. Agglutinins develop during the first 2 or 3 weeks. A positive agglutination in individuals who have been constantly exposed to infection (veterinarians, farmers, meat handlers, and tanners) may be of no significance since many of these individuals show agglutinins in the absence of any clinical signs of diseases.

Apparently a considerable number of persons have some acquired immunity.

c. **DIAGNOSTIC CRITERIA.** The isolation of small, gram-negative coccobacilli showing rather sparse growth, fermenting no carbohydrates, and agglutinating in a *Brucella* antiserum indicates the genus. Blood cultures should be examined periodically for 4 weeks before being discarded as negative. If carbon dioxide is required for growth, the organism is probably *Br. abortus*. Under ordinary circumstances, no further species differentiation is indicated.

d. **SPECIAL METHODS.**

- (1) *Microscopic.* The three strains are indistinguishable morphologically, however stained smears from pathological lesions should be examined for the small Gram-negative rods described above.
- (2) *Cultural.* Although the organisms may be found in the blood early in the disease and during the febrile periods and in urine and milk specimens at irregular intervals, the percentage of positive cultures, even from proved cases, is low.
 - (a) Obtain a specimen consisting of 10 to 12 ml of blood or 50 ml of urine or milk. Other substances such as the contents of an ovarian cyst, synovial fluid, and excised lymph nodes may also be subjected to cultural study.
 - (b) Inoculate two flasks containing 100 ml of 2 percent Trypticase Soy Broth with 5 to 10 ml of blood. A culture assembly which will reduce the chances of infection in laboratory personnel is described in paragraph 272i(2). Several loopfuls of sediment from a catheterized urine specimen, or sediment and cream layer from milk may be similarly inoculated. Also streak the specimen on two Trypticase Soy Agar plates.
 - (c) Incubate one set of media in incubator at 37° C. for growth of *Br. melitensis* and *Br. suis*; place the other set in jar containing 10 percent carbon dioxide and incubate at 37° C. for *Br. abortus*.
 - (d) Examine plates and gram-stained films from broth after 48 hours and at frequent intervals thereafter for growth. Streak new plates from broth at least once per week, even if no evidence of growth is discernible. Observe cultures for at least 4 weeks before reporting them as negative.
 - (e) Identify any positive culture as belonging to this

group by agglutination with antisera prepared against *Br. abortus*.

- (f) Although not usually required, the species of young cultures can be determined by tests for hydrogen sulfide production and by ability of the organism to grow on media containing certain dyes (basic fuchsin and thionin). (See table XXXIV.)

e. ANIMAL INOCULATIONS. *Br. melitensis* and *Br. suis* and less constantly *Br. abortus*, may be isolated from infected material by subcutaneous inoculation into guinea pigs (preferably males). After 4 weeks, kill the animal; examine gram-stained smears from the lymph nodes, spleen, and liver, and make cultures from the liver, spleen, blood, and lymph nodes. This test is seldom used because of the great danger of laboratory infection.

f. SEROLOGICAL.

- (1) *Identification of organisms.* There is complete cross agglutination in titer between an antigen prepared with any of the three species and antisera prepared against any other species. It is therefore unnecessary to maintain cultures or antiserum for more than one species.
- (2) *Identification of antibodies.* Serum from a patient taken after the fifth day of disease usually contains agglutinins. Set up macroscopic agglutination tests in dilution from 1:20 to 1:160 or higher against a *Br. abortus* antigen and against *Pasteurella tularensis* antigen. Agglutination of the *Brucella* antigen in a dilution of 1:100 or higher is considered to be significant. Cross-agglutination in serum from patients with brucellosis or tularemia is frequently present, but is less marked with the heterologous antigen. Agglutinins may persist for years after recovery. This is the most valuable test for diagnosing *Brucella* infections and is the only one routinely used. *Brucella* antigens vary in agglutinability. A standardized antigen should be employed. Previous vaccination for cholera has been shown to cause cross reaction with *Brucella* antigen due to homologous antigens contained in the two groups.

361. Genus *Pasteurella* (Hemorrhagic Septicemia Group)

This genus is characteristically pathogenic for lower animals, but it includes two species also pathogenic for man, *P. pestis* and *P. tularensis*. All species show bipolar staining. Milk is not coagulated. The genus can be divided as follows: The animal pasteurellas (five species) grow on ordinary media, produce indol

and hydrogen sulfide, and ferment sorbitol; they do not grow in bile. *P. pestis* grows on ordinary media, produces neither indol nor hydrogen sulfide, grows in bile, and does not ferment sorbitol. *P. tularensis* does not grow on ordinary media.

362. *Pasteurella Pestis* (Plague Bacillus)

a. CHARACTERISTICS. These are short, thick, nonmotile, gram-negative rods showing bipolar staining and pleomorphism that may be marked in direct smears of infected tissue. Characteristic bladder, safety-pin, and ring involution forms occur. Halos, indicative of the presence of capsules, may be seen in such smears. Growth on ordinary nutrient media is slow. Pleomorphic forms appear in old cultures or especially in cultures on 3 percent salt agar. In 24 hours on plain agar the colonies are 0.5 mm or less in diameter; in 3 to 4 days they reach a size of 3 to 4 mm and are grayish-yellow and viscous. In nutrient broth there is little turbidity but a granular deposit develops. The organisms are aerobic and grow poorly anaerobically. Biochemical reactions are of value in classification: *P. pestis* grows in litmus milk with no change in reaction or slight acid without clot, produces neither indol nor hydrogen sulfide, grows in bile, and fails to ferment sorbitol. When sugars are fermented, little acid is produced (pH 6.5) and no gas is formed.

b. HOST RELATION. *P. pestis* is the cause of plague in man and of a widespread disease of rats, ground squirrels, and other rodents. Two forms of plague occur in man: the bubonic type, contracted from infected rat fleas, and the pneumonic type, spread by sputum droplets from man to man. In bubonic plague, the infection first appears in the lymph nodes, usually those in the inguinal region. The organism may be obtained from these by puncture with a hypodermic needle. Infection is not restricted to this site since organisms may often be cultivated from the blood stream at any time during the disease. In some cases organisms may be seen in direct smears of the blood. The urine may, but the feces does not, contain plague bacilli. In the pneumonic type, the sputum contains the organisms in large numbers. In naturally infected rats, ground squirrels, and other rodents, similar lymph nodes or buboes may appear, which contain the organisms in large numbers; the spleen is also heavily infected. The bacilli may live for months in the bodies of dead animals.

c. SPECIAL METHODS.

- (1) *Microscopic examination.* Stain films from suspect materials by the Gram method, and with methylene blue,

crystal violet, or dilute carbol-fuchsin (for bipolar staining). The presence of gram-negative, short, ovoid, polar-staining bacilli, including many degenerated and poorly stained forms, is suggestive but not conclusive evidence of *P. pestis* infection.

- (2) *Culture*. Inoculate the surface of blood agar, glycerol agar, and 3 percent sodium chloride agar plates. Plant a sample of blood into infusion broth or Trypticase Soy Broth. Incubate cultures at 30° to 35° C. for 36 to 48 hours. Observe growth and transfer to differential media in accordance with characteristics given in *a* above.
- (3) *Animal inoculation*. Inoculate guinea pigs or mice subcutaneously with small amounts of the original specimen, or with a loopful of suspected culture. Putrefied materials may be applied to the freshly shaven abdomen of a guinea pig (plague bacilli penetrate the abraded skin, contaminants do not). If *P. pestis* is present, the animals will develop characteristic lesions and die in 2 to 5 days, with a characteristic post-mortem appearance. Subcutaneous injection into guinea pigs provokes local edema followed by inflammatory swelling of the regional lymph nodes and a generalized infection. At post mortem, the lymph nodes are enlarged and surrounded by hemorrhagic exudate. There are small, grayish, necrotic areas in the liver and spleen, and the bacilli are found in local lesions, buboes, and internal organs, especially the spleen and blood. *P. pestis* may be cultured from the lesions.

Caution: Animals should be freed of all ecto-parasites, prior to use, by dipping them in an antiseptic solution. Then place in glass jars covered with fine mesh gauze to prevent access or escape of any parasites. When handling animals, living or dead, protect the hands and arms by wearing rubber gloves and long-sleeved gown.

d. DIAGNOSIS OF PLAGUE IN RODENTS.

- (1) The natural infection in rodents can usually be detected by the post-mortem appearance, which includes a bubo, with hemorrhagic spots and areas of gray necrosis, subcutaneous and general congestion, a granular liver, with punctate hemorrhage and gray-yellow spots, a congested spleen, and pleural effusion.
- (2) Bacilli may be found in the bubo, liver, spleen, and blood, and isolated for study in pure culture by the methods used for human materials.
- (3) If the animal is to be shipped to a distant laboratory for

examination, the entire carcass is placed, without preservative, in a tightly sealed container that is packed in a second container to avoid breakage and escape of contents. The package must be shipped by express (Federal laws prohibit the shipment of plague-infected materials by mail). Decomposition may be avoided by surrounding the inner container with ice or "dry ice." Label the package "Perishable—for bacteriological examination—please expedite."

363. *Pasteurella Tularensis*

a. CHARACTERISTICS. This organism is an extremely small, gram-negative, nonmotile bacillus, bacillary, coccoid, and pleomorphic forms occur. It is best examined in preparations stained with crystal violet or carbol-fuchsin to demonstrate bipolar staining. No growth occurs on ordinary nutrient media unless 0.05 to 0.10 percent cystine is added. Growth is best on dextrose-cystine-blood agar, with small, gray, translucent colonies developing in 24 to 48 hours at 37° C. The organism is aerobic. It is serologically related to the genus *Brucella*, agglutinating in *Brucella* anti-serum to about one-fourth the homologous titer. The organism is exceedingly virulent, and many laboratory infections have occurred.

b. HOST RELATION. *P. tularensis* is the causative agent of tularemia, a natural disease of wild rabbits and ground squirrels. In man, the organism can enter the unbroken skin or be introduced by the bite of infected ticks or flies. Tularemia in man is usually contracted from handling or dressing infected rabbits. A local infection is set up, which spreads to give rise to a generalized infection.

c. SPECIAL METHOD.

- (1) *Microscopic examination.* This is of value for studying the morphology of the organisms (stained smears from the local ulcer may be negative) and to rule out *M. tuberculosis* by observing acid-fast stains of spreads made from pathological materials.
- (2) *Culture.* A sample of infected tissue, pus, fluid, or blood is planted on slants of dextrose-cystine-blood agar, and incubated at 37° C. for 3 to 5 days. Blood-agar plates are also planted to detect other organisms. Observe the cystine slants for characteristic colonies. If negative, continue observation for 21 days. If growth occurs, identify the organism by stained spread, pure culture transplants, and by macroscopic agglutination tests with a

high titer antiserum. Cultures made from the blood and lesions of man are usually unsatisfactory. Cultures should be made from the heart's blood, spleen, lymph nodes, and liver of guinea pigs following inoculation with material from the patient.

- (3) *Animal inoculation.* Inoculate guinea pigs, rabbits, or mice with suspected materials from lymph nodes, ulcers or blood, subcutaneously; if other bacteria are present, the material can be rubbed on the recently shaven, abraded abdomen. If *P. tularensis* is present, the animal usually dies in 5 to 10 days with characteristic lesions which include hemorrhagic edema, but no pus, at the site of inoculation, cervical axillary, or inguinal buboes, enlarged lymph nodes filled with dry, yellow, caseous material, an enlarged, dark spleen and a liver that contains discrete, white, caseous granules; organisms can be seen in spreads and can be cultured from the spleen, liver, bubo, and blood.

Caution: Extraordinary precautions must be taken in handling animals inoculated with *P. tularensis* because of the great risk of infection.

- (4) *Agglutination reaction.* The macroscopic tube method is preferred. Set up agglutination tests of the patient's serum against *P. tularensis* and *Brucella abortus* antigens. Agglutination of *P. tularensis* by serum in dilutions of 1:80 or higher is considered diagnostic of tularemia provided there is not a high cross-agglutination with *Brucella*. Agglutinins appear in the patient's blood after the first week of the disease and usually increase rapidly. Identity of a suspected culture may be established by a similar test, using a suspension of the organisms and serial dilutions of a *P. tularensis* antiserum of known titer. The resultant agglutination to be significant, must be present in dilutions approaching the known titer of the serum.

364. *Malleomyces Mallei* (*Pfeifferella Mallei*, Glanders Bacillus)

a. **CHARACTERISTICS.** The glanders bacillus is a small, slender rod, sometimes slightly curved, gram-negative, nonmotile, without spores, and nonacid fast. In culture, long filaments may appear. Growth occurs on ordinary nutrient agar with a 24-hour colony about 1 mm in diameter, smooth, grayish-yellow and translucent. A ropy sediment develops in broth. There is yellow to

brownish honeylike growth on potato. Milk is coagulated slowly and sometimes digested. Carbohydrates usually are not fermented. Some strains produce small amounts of acid from dextrose. Under anaerobic conditions almost no growth takes place.

b. **HOST RELATION.** *M. mallei* is the cause of glanders or "farcy" in horses, mules, and occasionally man. The disease may arise in an infected skin abrasion or from the infection of the nasal mucosa. Multiple subcutaneous abscesses appear from which the organisms may be demonstrated microscopically or culturally. The purulent nasal discharge which may be present will also contain the organisms. In smears of pus from older abscesses, it is often difficult to find the organisms, but cultivation is still possible.

c. **SPECIAL METHODS.**

- (1) *Animal inoculation.* Inject a small amount of infected material from a lesion, or a suspension of a culture, intraperitoneally in a male guinea pig. If *M. mallei* is present, orchitis starts in 2 to 3 days; later there is tumefaction and pus formation (the Strauss reaction). Post-mortem findings, in addition to the testicular lesions, consist of subcutaneous abscesses and small miliary grayish-white nodules in the liver, spleen, pancreas, and lungs. Bacilli may be recovered from these lesions.
- (2) *Complement fixation test.* This test can be applied to blood serum, using a special glanders antigen.
- (3) *Culture.* Aseptically removed specimens of pus from suspected lesions, lymph nodes, or other material, are placed on 3 percent glycerol agar (pH 6.6 to 7.0) and potato medium, and given prolonged incubation at 37° C. If *M. mallei* is present, after several days or a week, round, whitish or yellowish colonies appear; on potato media the growth is yellowish, semitransparent and honeylike, becoming brownish or amber and tenaceous, the medium assuming a green or greenish-brown tint.
- (4) *Histological sections.* Stained sections of infected tissue show typical glanders tubercles.

365. Genus *Hemophilus* and Differential Growth Requirements

a. **GENERAL.** The important pathogenic species of this genus are *H. influenzae*, *H. pertussis*, *H. ducreyi*, and *H. duplex*. They are small, nonmotile, nonspore forming, gram-negative rods, sometimes almost coccoid and sometimes threadlike and pleomorphic. They stain faintly, best with dilute carbolfuchsin. All are strict

parasites. They cannot be cultured on ordinary nutrient media, growing best (or only) in the presence of hemoglobin, and in general requiring blood, ascitic fluid, or other growth accessory substances found in plant or animal tissue. They grow best aerobically, but will grow poorly under anaerobic conditions.

b. METHOD OF TESTING GROWTH REQUIREMENTS. A simple test for determining the growth requirements of members of the *Hemophilus* group may be performed as follows:

- (1) Draw a line with a wax pencil across a pancreatic digest of casein agar or veal infusion agar plate to separate it into halves.
- (2) Streak one half of the agar with the culture to be tested.
- (3) Place a drop of blood on the other half, mix some of the culture to be tested in the drop, then streak over this half.
- (4) At right angles to these inoculations and across the center of the agar make a single streak with a culture of *Staphylococcus aureus*. Begin the streak on the side containing no blood and extend it across the side with blood. Incubate.

c. INTERPRETATION. The growth requirements of various *Hemophilus* organisms is indicated as follows:

- (1) Growth on the agar without blood, and not merely alongside the staphylococcus streak, indicating that neither X nor V factors are required:
 - (a) *H. pertussis*.
 - (b) *H. duplex*.
- (2) Growth on the agar without blood, but only alongside the staphylococcus streak (satellitism), indicating that only V factor is required:
 - (a) *H. parainfluenzae*.
 - (b) *H. hemolyticus* (markedly hemolytic; see also (4)(c) below).
- (3) Growth only on the agar with blood but without satellitism. Only the X factor is required.
 - (a) *H. ducreyi*.
 - (b) *H. canis* (*haemoglobinophilus*).
- (4) Growth only on the half with blood and showing distinct satellitism. Both X and V factors are required:
 - (a) *H. influenzae*. (Usually produces indol. Quellung reaction occurs with type-specific antisera.)
 - (b) *Koch-Weeks' bacillus*. (Produces no indol. Agglutinates human erythrocytes, spot test using 0.5 percent suspension of red cells and broth cultures.)

- (c) *H. hemolyticus*. (Markedly hemolytic; some strains do not require X factors—see (2)(b) above.)
(d) *H. suis*. (Ferments maltose and sucrose.)

366. *Hemophilus Influenzae* (Influenza Bacillus, Pfeiffer's Bacillus, Koch-Weeks Bacillus)

a. CHARACTERISTICS. For growth, the influenza bacillus requires two accessory substances, the so-called "X" and "V" factors. X is furnished by hematin, a break-down product of hemoglobin. V is a co-enzyme present in blood and various vegetable and animal tissues. Heated-blood (chocolate) agar or heated-blood broth contains both factors and is the medium of choice. Subcultures on plain or serum agar fail to grow. A 24-hour colony on chocolate agar is a little less than 1 mm. in diameter and is translucent. Some strains also will grow on ordinary blood agar but usually not well. Fair growth or small nonhemolytic colonies occurs in 48 hours on unheated rabbit or sheep blood agar. There is less growth with human blood and no growth with cow or goat blood. If the blood is heated, as in chocolate blood agar, the species of blood is unimportant. On ordinary blood agar plates with a variety of growth, the colonies of *H. influenzae* (or of any species of *Hemophilus* requiring the V factor) in close proximity to the colonies of certain other bacteria (particularly staphylococci) are much larger than those not so located; this phenomenon is due to the liberation of a co-enzyme by the contaminating bacterium. Indol is produced, nitrates are reduced and dextrose is fermented in heated-blood broth. Other fermentation reactions are of little differential value.

b. HOST RELATION. The influenza bacillus is a common inhabitant of the normal respiratory tract and is often present in large numbers in tuberculous sputum. Sometimes it is involved in secondary pneumonia, particularly during an influenza epidemic, although it is not the primary cause of influenza, as was once believed. It is a frequent cause of non-epidemic meningitis, and in such cases the organisms may be seen in direct smears of the cerebrospinal fluid and may be cultivated from it. It is in this condition that identification is usually requested. The influenza bacillus may also be responsible for conjunctivitis, and was first described in this connection as the Koch-Weeks bacillus.

c. IDENTIFICATION.

- (1) *Conjunctival specimens*. Make a slide spread from the conjunctiva and stain by the Gram method and with dilute carbol-fuchsin. Observe for minute gram-negative

bacilli, often intracellular. Cultures are not informative except to reveal other organisms.

- (2) *Spinal fluid, sputum, and other materials.* Culture on chocolate agar, and incubate at 37° C. for 2 days. Colonies are identified by their appearance, the microscopic morphology of the organisms and the failure of subcultures to grow on plain agar. The colony may be confused with that of a streptococcus. Specific identification considers the source of the specimen, its hemolytic properties, and the requirements of accessory growth factors.

367. *Hemophilus Hemolyticus*

This bacillus is of little or no pathogenic significance, but is sometimes found in the upper respiratory tract. It produces beta hemolysis in unheated rabbit blood agar, and thus may be mistaken for a beta-hemolytic streptococcus. The growth requirements are about the same as those of *H. influenzae*.

368. *Hemophilus Pertussis*

a. CHARACTERISTICS. Small, gram-negative rods of considerable regularity and with little tendency to form filaments are characteristic of this organism. Although the X and V factors are not required, for primary isolation a special blood glycerol potato agar (pH 5.0) (Bordet-Gengou medium) must be used. The plates should be incubated in a moist atmosphere for 4 or 5 days before being discarded. At some time during this period very small, almost globular, colonies may appear; such colonies have a slightly grayish luster by reflected light and have been compared to droplets of mercury. Later there is a hazy zone of hemolysis about the colony, difficult to see because of the large amount of blood in the medium. After continued subcultivation, the organism usually grows on ordinary blood agar and, eventually, on unenriched nutrient agar, but such a change is accompanied by dissociation and the loss of the antigenic properties that are desirable for the making of efficient vaccines. Dextrose is not fermented, indol is not formed, and nitrates are not reduced. Very rarely, *H. parapertussis*, primary growth of which will occur on ordinary nutrient agar, may be encountered.

b. HOST RELATION. As the cause of pertussis (whooping cough), *H. pertussis* is present in large numbers in the trachea and bronchi during the first 2 or 3 weeks of the disease but is difficult to isolate after that time. Isolation may be done by the cough-plate

method, in which a plate of Bordet-Gengou medium is held vertically in front of, and a few inches away from, the face of the patient during a paroxysm of coughing. Swabbings, taken from the nasopharynx, often yield positive cultures more frequently than do cough plates. Isolation of *H. pertussis* is aided by incorporating 0.3 unit of penicillin into each ml of medium.

c. DIAGNOSTIC CRITERIA. The presence of very small, gram-negative, rather regular rods, failing to grow on ordinary media on first isolation, producing small grayish colonies with hazy hemolysis in or after 72 hours on Bordet-Gengou medium establishes the probable identity.

369. *Hemophilus Ducreyi* (Ducrey's Bacillus)

a. CHARACTERISTICS. These are small, short, gram-negative rods that may have ovoid forms, chain formation is occasionally seen. In direct smears from lesion, the organisms may appear within leucocytes. Cultivation is difficult; either a small amount of whole rabbit blood, allowed to clot in a small test tube and heated at 56° C. for 15 minutes, or blood agar made with 20 to 30 percent defibrinated rabbit blood, usually gives satisfactory growth. The 24-hour colony at 37° C. is usually less than 1 mm in diameter, grayish, and granular.

b. HOST RELATION. This organism is the causative agent of chancroid, or soft chancre, a venereal infection that is not inoculable to lower animals. The primary lesion is a spreading ulcer; smears made from the lesion show the characteristic organisms. Cultures may be made directly from the lesion. The regional lymph nodes are enlarged. From these buboes material for cultures may be obtained by aspiration with a sterile hypodermic syringe. From the latter source pure cultures are likeliest to be obtained.

c. DIAGNOSTIC CRITERIA. A bacteriological diagnosis of chancroid is difficult and is not often necessary. It is sufficient to demonstrate characteristic, gram-negative rods in direct smears and in cultures from the ulcers or buboes, as indicated above. Only the X factor is required by *H. ducreyi*.

370. *Hemophilus Duplex* (*Moraxella Lacunata*)

a. CHARACTERISTICS. This organism is broader and longer than the other members of the genus and tends to be arranged in pairs, resembling Friedländer's bacillus. It is not encapsulated. Growth is best on blood or serum containing medium. Twenty-four hour colonies are gray, translucent, and about 1 mm in diameter. In 3 to 4 days they reach a diameter of 5 to 6 mm. Growth is less

abundant on chocolate agar. On Loeffler's coagulated serum, slight liquefaction occurs with pitting of the surface under each colony.

b. HOST RELATION. *H. duplex* is the cause of a low-grade conjunctivitis.

c. DIAGNOSTIC CRITERIA. Direct smears of conjunctival pus should show thick, short, gram-negative rods, commonly arranged in pairs. They are free or within pus or epithelial cells. The characteristic growth on blood agar plates, the absence of growth on ordinary media, and slight liquefaction of Loeffler's coagulated serum should be demonstrated.

371. Genus *Bacillus* (Aerobic Spore Formers)

This comprises a large group (146 species) of gram-positive, spore-bearing organisms. Only one species (*B anthracis*) is pathogenic, but the others are frequently encountered as contaminants. One of these, *B. subtilis*, will be described as representative.

372. *Bacillus Subtilis* (Hay Bacillus)

a. CHARACTERISTICS. This organism is a long, actively motile, gram-positive rod. Gram staining may be uneven, with gram-positive granules appearing. Short chains usually occur. The spore, which is seen as an ellipsoidal, refractile, unstained body, is usually centrally located and does not bulge beyond the cell borders. Growth occurs readily on ordinary nutrient media. The colony on agar is characteristically rough and dry. In broth a surface pellicle appears. Gelatin is liquefied, and coagulated serum is digested. Growth is aerobic but also occurs in an atmosphere containing reduced oxygen. The spores are extremely resistant, withstanding boiling for hours; they are killed by autoclaving at 120° C. for 15 to 20 minutes.

b. HOST RELATION. The organism is essentially nonpathogenic and is commonly present in dust and soil, although it may appear in old wounds as a saprophyte and be confused with *B. anthracis*. It is sometimes the cause of a severe conjunctivitis.

373. *Bacillus Anthracis*

a. CHARACTERISTICS. Anthrax bacilli are large, nonmotile, gram-positive, sporulating rods. In blood and body fluids of infected animals, they occur in pairs or short chains. In cultures, they occur in long, segmented, parallel chains. The ends of the bacillus are usually square or concave. Capsules are formed in the animal body and on serum media, but are lost on agar. Spores are centrally placed, formed only in the presence of oxygen, and

are not formed in the animal body. On an agar plate, the colony is large, raised, dull, opaque, and grayish-white, with irregular borders and an uneven surface; it has so-called "medusa-head" appearance under the low power of the microscope. In a gelatin stab there is a crateriform liquefaction, with an inverted pine tree appearance. Growth is aerobic and facultatively anaerobic. Spores are killed by boiling for 10 minutes, but may survive in a dry state for years.

b. **HOST RELATION.** The anthrax bacillus is a natural pathogen of cattle, sheep, and horses, giving rise to anthrax in these animals. The infection in man is usually a pustule on the skin. From this lesion, the organisms can be seen on direct smear, and can be cultivated. In the pneumonic form, which is sometimes seen, the organisms are contained in the sputum. Mice and guinea pigs are susceptible to infection by subcutaneous inoculation.

c. **DIAGNOSTIC CRITERIA.** The material to be examined may consist of pus or fluid from skin lesion (malignant pustule), blood in the septicemic stage of a diseased or infected animal, sputum from a case of pulmonary infection, and spinal fluid from a case of meningeal infection (rare).

- (1) *Microscopic examination.* Make film preparations with the infected material, stain by the Gram method, and examine for the characteristic, large, gram-positive bacilli. Spores may be present only if the bacilli have been exposed to an atmospheric concentration of oxygen. In blood or animal tissues, the organisms are encapsulated.
- (2) *Culture.* Plant portions of the specimen in nutrient broth and on agar plates. Incubate at 37° C. for 24 hours or more, and observe colonies. For pure culture isolation, heat the broth culture to 60° C. for 20 minutes to kill the associated organisms, and transplant on agar plates.
- (3) *Animal inoculation.* This is an important diagnostic procedure. Inoculate white mice, guinea pigs, or rabbits subcutaneously with small portions of the broth culture or of a suspension of the agar growth, or, for a rapid diagnosis, the original suspected material. If anthrax bacilli are present, the animal will die with a fatal septicemia in from 12 to 72 hours. The blood will be dark and swarming with the square-ended bacilli. The organisms may be isolated from the blood, liver, and spleen.
- (4) *Diagnosis.* The diagnosis of anthrax is warranted if the specimen contains a gram-positive, square-ended, chain-producing spore-forming, nonmotile bacillus that produces characteristic medusa-head colonies on agar and,

when injected subcutaneously into small animals, produces a fatal septicemia.

374. Genus *Clostridium* (Anaerobic Spore Formers)

a. GENERAL.

- (1) The clostridia are a wide-spread group of organisms, being found in comparatively large numbers in soil, dust, and fecal material. Because of their ability to form spores, they are able to survive influences which are generally fatal to most non-spore-forming organisms and, consequently, they are to be found in any site which has been exposed, directly or indirectly to contact with dust, soil, or fecal material. Although sixty-one species are now listed, only a comparatively small number are of medical importance—primarily the members of the “gas gangrene group” and *Cl. tetani*, *Cl. botulinum*, and *Cl. para-botulinum*.
- (2) Clostridia are very commonly found in wounds which have been contaminated with dust, soil, or clothing. The presence of pathogenic species in a wound is not an occasion for alarm, but is rather to be expected, for between 20 and 40 percent of war wounds examined in various surveys have been found to contain clostridia. These organisms usually rapidly disappear from the wound, but in a few cases, rapid and highly fatal infections develop. It is with these cases, and with the organisms which can give rise to them, that the medical bacteriologist is primarily concerned.
- (3) Although the isolation and identification of spore-forming anaerobic bacteria from infected wounds is not usually as difficult as is commonly considered, it is often time-consuming and tedious. Furthermore, the interpretation of the results is not simple, for these organisms are so commonly encountered that the demonstration of a member of a pathogenic species in a wound does not necessarily indicate that active invasion of the tissues by the organism is occurring, or even that it is likely to occur. Furthermore, since the distribution of these organisms in the infected tissue is not uniform, the demonstration of one pathogenic species does not necessarily indicate that it is the only one involved, nor does the lack of demonstration of a given species necessarily assure its absence. The fact that gas gangrene is a clinical entity and not a bacteriological entity is often insufficiently appreciated.

b. COLLECTION AND CULTURE OF SPECIMENS.

- (1) Tissue fragments taken from suspected wounds by surgical removal or on a sterile cotton swab are placed in tubes of sterile chopped meat medium (par. 272r) or thio-glycollate medium (par. 268e).
- (2) The tubes are subcultured immediately to egg-yolk agar plates (par. 275k) and to blood agar plates. If blood from a blood bank is used in the making of blood agar, sufficient calcium chloride (5 ml of 5 percent anhydrous CaCl_2 per 100 ml of blood) should be added to the medium to combine with the citrate used as an anti-coagulant, for unless a slight excess of free calcium is present the hemolytic patterns of some species will not be typical. It is usually advisable, also, to use at least 3 percent agar in the blood agar medium to lessen the possibility of swarming—a characteristic that is quite marked with certain species, such as *Cl. novyi*. The drying of the surface of the agar by incubation prior to inoculation also is helpful. Incubation should be at 35° to 37° C., preferably in an anaerobic jar, for 48 hours (see pars. 268 and 269).
- (3) Representatives of all colony types are picked, examined microscopically, and inoculated into stock semi-solid medium,² which is incubated overnight. If it is at all possible, replating from the semi-solid medium should be done before differential media are inoculated, for the anaerobic spore-formers are more difficult to obtain in pure culture than are the aerobic or facultative bacteria. *If only the pathogenic species of clostridia are to be isolated and identified, only those colonies exhibiting hemolysis on blood agar plates need to be examined, for all of the pathogenic species are hemolytic.*

c. PRESUMPTIVE IDENTIFICATION. Presumptive identification of some species is often possible by the use of egg-yolk agar. On this medium, after 48 to 72 hours of incubation, the precipitation patterns of some species are fairly characteristic. Identity should always be confirmed, however, by more detailed study.

- (1) *Cl. perfringens*. Colonies are round and smooth, and surrounded by a wide (5 to 10 mm) zone of opaque white precipitate. There is no luster.
- (2) *Cl. novyi*. Colonies are smooth, and often thin, with irregular edges. A precipitation zone is seen under the

²This is the medium given in par. 271e(1) with added dextrose 0.1 percent and dipotassium phosphate (K_2HPO_4) 0.1 percent. The phenol red is not necessary. Be sure to heat in boiling water for 5 minutes then cool rapidly, just before using.

colony and around it for 3 to 5 mm. The precipitation is more intense than with *Cl. perfringens*, the edge of the zone is sharply defined, and the colony and some of the surface of the agar is covered by an iridescent luster.

- (3) *Cl. bifermentans*. Colonies are small to medium size with slightly raised, often eroded margins and with a wide zone of precipitation. No luster is seen. These colonies resemble *Cl. perfringens*, but *Cl. bifermentans* forms spores readily, while *Cl. perfringens* rarely does so.
- (4) *Cl. sporogenes*. The precipitate is primarily under the colony and rarely spreads beyond. A slight luster is usually found on the colony.
- (5) *Clostridium parabotulinum*. (Types A and B). Colonies are raised with irregular margins. Precipitation is seen under the colony and in a narrow zone around it. Luster covers the colonies and the precipitation area.
- (6) *Cl. botulinum*. (Types C, D, E). Colonies are flat, irregular and surrounded by a wide zone of precipitation. Luster covers the colony but does not extend to the edge of the precipitation zone.
- (7) It should be noted that certain pathogenic species do not cause any precipitation of the egg-yolk agar. These species are *Cl. tetani*, *Cl. septicum*, *Cl. histolyticum*, *Cl. fallax*, *Cl. carnis* and *Cl. difficile*.
- (8) Since a number of species of the genus *Bacillus* will give precipitation on egg-yolk agar, it is well to test each colony type for catalase (see par. 296). Catalase-positive organisms, such as members of the genus *Bacillus*, cause the more-or-less rapid evolution of bubbles of oxygen from the hydrogen peroxide; the catalase negative clostridia do not do so.
- (9) Hemolytic and egg-yolk agar reactions are given in table XXXV.

d. CONFIRMATORY TESTS. The day before differential media are to be inoculated, tubes of stock semi-solid media should be inoculated from isolated colonies on blood agar or egg-yolk agar plates. After overnight incubation, the differential media are inoculated from the stock semi-solid cultures. It is generally desirable to use a pipette, rather than a loop for such inoculation, transferring 0.1 to 0.2 ml of the semi-solid culture to the bottom of each tube of differential medium. Differential media in addition to blood agar and egg-yolk agar are as follows:

- (1) *Iron-gelatin*. See paragraphs 274f, and 286.
- (2) *Indol test medium*. Inoculate the medium given in paragraph 274h and place the tubes in an anaerobic jar. The

reaction is read in accordance with instructions given in paragraph 283. Some have used this medium with added 0.2 per cent di-sodium phosphate (Na_2HPO_4) and 0.1 percent of each of the following: sodium thio-glycollate, agar and dextrose, to preclude incubation in an anaerobic jar.

- (3) *Sugar-free base for fermentation tests.* (See par. 271e.) Add individual sugars in accordance with instructions given. Incubation in an anaerobic jar is not necessary if the oxygen is eliminated prior to inoculating by placing in a boiling water bath and cooling rapidly, according to the instructions. If acid production is doubtful, remove a loopful of culture from the semi-solid fermentation medium and mix with a drop of 0.04 percent aqueous brom-thymol blue. Acidity is indicated by a yellow color.

- (4) *Litmus milk.* (See paragraphs 274g and 282.)

e. **RAPID IDENTIFICATION.** A number of short-cut methods have been described for the rapid identification of certain pathogenic clostridia, usually in mixed culture. The first two of these methods often give misleading results:

- (1) The so-called "stormy fermentation" of milk, which is sometimes stated to be characteristic only of *Cl. perfringens*, is given by a number of other clostridia and should never be employed by itself for the identification of this species.
- (2) The inoculation of guinea pigs with *mixed* primary cultures, protecting one guinea pig with *Cl. perfringens* antitoxin, another with *Cl. novyi* antitoxin, etc., is another method which often yields unsatisfactory results, for two pathogenic species commonly occur in the same infection. When this happens, all the guinea pigs perish, and no worthwhile information is obtained.
- (3) A rapid method for identifying *Cl. perfringens* is now widely used. It depends upon the inhibiting action of antitoxin on the formation of opacity on egg-yolk-agar or 20 percent serum agar medium. One-tenth ml of *Cl. perfringens* antitoxin (50 to 100 international units) is spread over half the surface of the medium. After the antitoxin has been absorbed, the inoculum is sown equally on both halves. The plates are incubated anaerobically 24 to 48 hours. Great care must be taken to spread an even layer of antitoxin exactly to the central dividing line and to the periphery of the agar, and to leave uninoculated a few millimeters at the edge of this "control" half; otherwise colonies with zones may occur at

the margin of the antitoxin treated area and confuse the reading of the test. Polyvalent gas-gangrene antitoxin should not be used.

- (a) Colonies of toxigenic *Cl. perfringens* produce in 24 hours marked zones of opacity; the formation of these zones is neutralized specifically by *Cl. perfringens* antitoxin.
- (b) Some streptococci and a few aerobic and anaerobic spore-bearers also give zones of opacity, but the formation is not inhibited by the antitoxin.
- (c) *Cl. sordelli* and *Cl. bifermentans* give zones specifically neutralized by *Cl. perfringens* antitoxin, but these reactions are usually feeble and these two organisms are readily distinguishable from *Cl. perfringens* by the presence of spores in stained smears of the 24 hour colonies as well as by their inability to ferment lactose (table XXXV).

f. FINAL IDENTIFICATION. The final identification of *isolated* strains of the pathogenic clostridia should include, whenever possible, pathogenicity tests in guinea pigs, including protection tests with monovalent antisera. While some species, such as *Cl. fallax*, easily lose pathogenicity in laboratory culture, the more important pathogenic species usually retain their pathogenicity for some time.

g. UNIDENTIFIABLE ORGANISMS. The biochemical and other important characteristics of the clostridia likely to be encountered in wounds are given in table XXXV. If an anaerobic spore-former is isolated which does not appear to be identical with any of those given, it should be remembered that from 5 to 15 percent of the clostridia isolated from wounds are usually unidentifiable.

h. ADDITIONAL INFORMATION. Additional information on the most commonly encountered pathogenic clostridia is given in the following paragraphs.

375. *Clostridium Tetani* (Tetanus Bacillus)

a. CHARACTERISTICS. These organisms are thin rods with spherical terminal spores. Filamentous forms are common. Colonies are small, greyish, translucent, with granular surfaces and villous to fimbriate edges, surrounded by a slight zone of hemolysis. There is a tendency to swarm on moist agar. (See table XXXV, and par. 374).

b. HOST RELATIONS. Tetanus spores are commonly present in the feces of man, horses, and cattle, or in soil contaminated with these materials. The spores may be introduced into wounds, and if

Table XXXV. Differentiation of *Clostridia*

	Hemolysis	Aerobic growth	Egg yolk agar reaction	Gelatin	Indol	Glucose	Maltose	Lactose	Sucrose	Salicin	Litmus milk	Spores	Pathogenicity
<i>Cl. carnis</i>	+	+	-	-	-	+	+	+	+	+	G	O,E	+
<i>Cl. tertium</i>	+	+	-	-	-	+	+	+	+	+	A,C,G	O,T	-
<i>Cl. histolyticum</i> ..	+	+	-	+	-	-	-	-	-	-	D	O,E	+
<i>Cl. perfringens</i> Type A.....	+	-	+	+	-	+	+	+	+	-	A,C,G	O,E	+
<i>Cl. novyi</i>	+	-	+	+	-	+	+	-	-	-	A	O,E	+
<i>Cl. bifermentans</i> ..	+	-	+	+	+	+	+	-	-	-	C,D	O,E	(+)
<i>Cl. sporogenes</i> ...	+	-	+	+	-	+	+	-	-	-	C,D	O,E	-
<i>Cl. fallax</i>	+	-	?	-	-	+	+	+	+	+	A,C,G	O,E	(+)
<i>Cl. lentoput-</i> <i>rescens</i>	+	-	?	+	-	-	-	-	-	-	C,D	S,T	-
<i>Cl. hastiforme</i> ...	-	-	?	+	-	-	-	-	-	-	C,D	O,E	-
<i>Cl. tetani</i>	+	-	-	+	+	-	-	-	-	-	C	S,T	+
<i>Cl. septicum</i>	+	-	-	+	-	+	+	+	-	+	C,G	O,E	+
<i>Cl. capitorale</i> ...	(+)	-	-	+	(+)	+	-	-	-	-	A,(C)	O,T	-
<i>Cl. sphenoides</i> ...	+	-	-	-	(+)	+	+	+	(+)	+	A,C	S,E-T	-
<i>Cl. tetano-</i> <i>morphum</i>	+	-	-	-	(+)	+	+	-	-	-	-	S,T	-
<i>Cl. difficile</i>	(+)	-	-	-	-	+	-	-	-	+	-	O,E	+
<i>Cl. butyricum</i> ...	-	-	-	-	-	+	+	+	+	+	A,C,G	O,E	-
<i>Cl. multifermen-</i> <i>tans</i>	+	-	-	-	-	+	+	+	+	+	A,C,G	O,E	-
<i>Cl. aerofetidum</i> ..	-	-	-	+	-	+	+	+	-	+	A,C,G	O,E	-
<i>Cl. botulinum</i>	+	-	+	+	-	+	+	-	-	-	A	O,E	+
<i>Cl. parabotulinum</i> (+)	-	+	+	+	-	+	+	-	-	(+)	(A),C,D	O,E	+

Reactions in parentheses are variable.

A—Acid, C—Clot, G—Gas, D—Digested, O—Oval, S—Spherical, T—Terminal, E—Eccentric (subterminal to near-central).

anaerobic conditions exist, such as in a deep, penetrating wound, growth will occur, especially when there is considerable tissue destruction. The organisms remain localized in the wound, producing a powerful toxin, which is absorbed into the system and causes the typical symptoms of tetanus.

c. DIAGNOSTIC CRITERIA. The identification of *Cl. tetani* in infected wounds is usually quite difficult. Although it may occasionally be demonstrated by microscopic or cultural methods, the most practical test is animal inoculation.

d. COLLECTION OF SPECIMENS. Pus and tissue fragments taken from suspected wounds by surgical removal, on a sterile cotton swab, or on a platinum loop, may be placed in a tube of sterile

saline solution, and this used for microscopic examinations, cultures, and toxicity tests.

e. **MICROSCOPIC EXAMINATION.** Make film preparations of the suspected material. Stain and examine for the characteristic drum-stick spores of *Cl. tetani*. If present in small numbers, they may be overlooked. If nonvirulent anaerobic or aerobic bacilli with round terminal spores are present, differentiation from the *Cl. tetani* cannot be made. Therefore, this method of diagnosis is of little practical value.

f. **CULTURE.** (See par. 374.)

g. **ANIMAL INOCULATION.** Mix a portion of the original material, of the heated culture (80° C. for 30 minutes to kill non-sporulating organisms) or, preferably, of a broth suspension of a pure culture, with an equal quantity of an irritant such as sterile 5 percent calcium chloride, or lactic acid, and inject 1.0 ml subcutaneously into the thigh of a guinea pig. A control pig receives the same injection plus an intraperitoneal inoculation of 1,000 units of tetanus antitoxin. If *Cl. tetani* is present, the unprotected animal will develop tetanus and die in 1 to 4 days.

h. **DEMONSTRATION OF TETANUS TOXIN.** Inject, subcutaneously, 0.5 ml of filtrate of a 10-day broth culture into each of two mice or guinea pigs, one of which has been given a prophylactic dose of antitoxin (intraperitoneal). The development of symptoms of tetanus in the unprotected animal proves the presence of toxin in the filtrate.

376. Organisms Associated With Gas Gangrene³

a. The anaerobic organisms associated with gas gangrene may be divided, on the basis of pathogenicity, into three groups:

- (1) *Pathogenic.* The organisms pathogenic for man are *Cl. perfringens (welchii)*, *Cl. septicum (Vibrio septique)*, *Cl. novyi (oedematiens)*, and *Cl. bifermentans (Cl. oedematoides or B. sordelli)*; that for animals is *Cl. chauvoei*.
- (2) *Slightly pathogenic.* The less pathogenic species are *Cl. histolyticum* and *Cl. fallax*.
- (3) *Nonpathogenic.* The nonpathogenic species are *Cl. sporogenes*, *Cl. aerofetidum*, *Cl. lento-putrescens (putrificum)*, *Cl. tertium*, and others.

b. On the basis of their biochemical reactions, they may be separated into a saccharolytic group and a proteolytic group. (See table XXXV.) There is not a strict demarcation of these proper-

³ An excellent account of the clostridia in gas gangrene is presented in Bacteriological Reviews, 13:233, 1949, by L. De S. Smith.

ties, for most members have some properties of the other group—that is, some are both saccharolytic and proteolytic but are classified according to the property that is most prominent. Most of the pathogenic group are saccharolytic. The organisms of the proteolytic group (except *Cl. histolyticum*) are not in themselves pathogenic but complicate wounds by their intense proteolytic action; they are saprophytes, have no power of invading the tissues and, if present without members of the saccharolytic group, usually do not interfere with the healing of the wound. None of the proteolytic clostridia produce a potent exotoxin, hence in infections caused by them there is no toxemia, in spite of the great liquefaction of tissue.

c. Cultures from wounds may contain both saccharolytic and proteolytic bacilli, which are difficult to separate by culture methods. Such a mixed culture may be purified by animal inoculation, since the pathogenic organisms of the saccharolytic group usually invade the blood stream and may be isolated from the heart's blood. *Cl. sporogenes*, the most frequent and active proteolytic clostridia, causes confusion by morphologically resembling *Cl. septicum*.

377. *Clostridium Perfringens* (*Cl. Welchii*)

a. These organisms are short, thick, gram-positive rods, *Cl. perfringens* is one of the few non-motile clostridia. Capsules are formed in the animal body and in culture (in sugar-containing media) if the pH is kept above 7. Sporulation is rare. On blood agar, colonies are round, 2 to 5 mm in diameter, greyish-white with an opaque raised center and usually surrounded by a double zone of hemolysis. *Cl. perfringens* often causes the coagulation of the agar in semi-solid media. Six types of this species are known, of which Types A and F are important in human medicine, Type A being responsible for many cases of gas gangrene and Type F for enteritis necroticans, a somewhat rare food poisoning disease. The types of *Cl. perfringens* are identified by the combination of toxins that are produced. There is much variation in toxigenicity and virulence of different strains. The bacillus is usually pathogenic for man and small animals, the latter dying following intramuscular injection, with extensive blood-stained fluid necrosis of the tissues, and marked gas formation; the muscles are friable and pale pink; the wound gives a foul acid odor, but there is no putrefaction.

b. The free fermentation of sugars is a prominent characteristic both in its production of gas gangrene and in the laboratory identification of cultures. *Cl. Perfringens* ferments the muscle

sugars, producing gas in the tissues; this forced along fascial planes and vessels, giving the crepitation of gas gangrene. Fermentation in the test tube may be so marked that the plug is blown out; in milk cultures it is made evident by "stormy fermentation"—an acid clot torn by gas bubbles and separation of the milk into coagulum and whey.

c. Gas gangrene due to *Cl. perfringens* is essentially a local infection, and the bacilli do not invade the blood stream until shortly before death. Spores are never formed in the animal body.

d. The toxin produced is an exotoxin comparable to that of tetanus and diphtheria. An effective antitoxin (see above) is used in therapy. This antitoxin is specific only for *Cl. perfringens*, not for other wound anaerobes; therefore, if gas gangrene associated with *Cl. perfringens* and *Cl. novyi* is treated by a monovalent antitoxin, the latter infection would not be influenced. Most commercial antitoxins, however, are polyvalent, thus affording protection against several forms of gas gangrene.

378. *Clostridium Novyi* (*Cl. Oedematiens*)

This is a long, thick, motile gram-positive rod which frequently swarms on the surface of the agar. Capsules are not formed. Sporulation is common. On blood agar, colonies are irregularly round, with undulate edges and granular surfaces. Slight hemolysis is seen beneath the colony. Lesions in experimentally infected guinea pigs are characterized by a whitish, often transparent gelatinous edema, little necrosis, and no gas. The bacillus forms a soluble toxin, which is used to prepare antitoxic sera.

379. *Clostridium Septicum* (*Vibrion Septique*)

a. These organisms are large slender motile gram-positive rods. They sporulate freely and do not produce capsules. Marked pleomorphism occurs in many strains, the cells having pale swollen bodies and pointed extremities. The occurrence of long filamentous forms in the livers of guinea pigs dying of this infection is characteristic. Invasion of the blood stream occurs. *Cl. septicum* is a strict anaerobe. Colonies are irregularly round, 5 to 8 mm in diameter, filamentous, translucent, and with fimbriate edges. Some strains show a marked tendency to swarm. Hemolysis occurs beneath the colony. The hemolysin and lethal toxins are identical in this species, and cause local necrosis and death in guinea pigs inoculated intramuscularly. The antitoxin is specific and does not protect against *Cl. perfringens* or *Cl. novyi*.

b. This bacillus is closely related and similar to *Cl. chauvoei*, the bacillus of symptomatic anthrax or "blackleg" of cattle and

sheep. The latter has never been isolated from wound cultures and has never been known to cause infection in man.

380. *Clostridium Bifermentans* (Cl. Sordelli, B. Oedematoides)

These are medium-size gram-positive rods, sporulating very freely, 48-hour colonies being sometimes composed entirely of free spores. This is the only anaerobic species in which spores in chains are commonly seen. Colonies on blood agar are ameboid to irregularly circular, 3 to 5 mm in diameter, greyish-yellow becoming dead white and opaque on sporulation, and are surrounded by a small zone of hemolysis. Some strains are pathogenic while others are not. The great majority of strains are not pathogenic to guinea pigs.

381. *Clostridium Sporogenes*

This species is very commonly found in wounds. It is composed of medium size gram-positive rods, sporulating freely. Colonies on blood agar are 2 to 5 mm in diameter, with raised, glistening centers, 0.5 to 1 mm in diameter, surrounded by tangled rhizoids—the so-called “medusa head” colony. These colonies tend to adhere to the agar. On moist agar, smooth lobate outgrowths from a rhizoid center are common. A small zone of hemolysis occurs around the colony. Although *Cl. sporogenes* is, by itself, only slightly if at all pathogenic, it appears to increase either the rate of growth *in vivo* or the pathogenicity of the toxigenic species.

382. *Clostridium Histolyticum*

These are medium to small gram-positive rods, sporulating freely. Colonies on blood agar vary from “dew-drop” transparent colonies 0.5 to 1 mm in diameter to irregularly round colonies, slightly raised, 1 to 3 mm in diameter with slight hemolysis. This species will grow feebly on aerobic plates. Pleomorphism is marked under such conditions and spores are seldom formed. An orange to wine-red color is produced in iron-gelatin medium; this characteristic is peculiar to this species. This organism seems to be pathogenic primarily because of its extremely potent proteolytic enzymes, which cause rapid and complete digestion of muscle and tendon.

383. *Clostridium Botulinum* and *Clostridium Parabotulinum*

a. These are rather large, thick, gram-positive, sluggishly motile bacilli, with an oval spore between the center and the end

of the cell that is broader than the cell. They are strictly anaerobic saprophytes. In blood agar or liver agar, the colonies attain a fair size. The growth of *Cl. paratubulinum* is usually more profuse than that of *Cl. botulinum*, surface colonies of the former often having a tendency to spread. *Cl. botulinum* probably corresponds to the strain originally isolated by Van Ermengem and is non-ovolytic (fails to digest coagulated egg or serum). *Cl. paratubulinum* is more frequently encountered in food poisoning of man and is ovolytic, digesting not only egg and serum but also meat and casein. Culturally, it resembles *Cl. sporogenes*. (See table XXXV for other characteristics.) There are many varieties of these organisms, differentiated chiefly by their production of specific toxins, most of which are neutralized only by their specific antitoxins. Such varieties are usually designated as types A, B, C, etc.

b. These anaerobes produce one of the most powerful toxins known. The toxin is formed not in the animal body but in improperly processed food. Unlike most other toxins, it is effective when taken by mouth. Botulism is not an infection but a true toxemia. The organisms washed free of toxin are harmless. The organisms are commonly present in soil. The toxin is destroyed by heating at 80° C. for 15 minutes.

c. The isolation from suspected food of a strictly anaerobic spore-forming bacillus that corresponds to the description given above and cultures of which are highly toxic when fed to mice or guinea pigs is presumptive evidence of *Cl. botulinum* or *Cl. paratubulinum*. The type of toxin can be determined only by the use of animals protected with various types of antitoxin. These antitoxins are not generally available. The symptoms of botulism in man are so characteristic that detailed bacteriological diagnosis is seldom requested.

384. *Corynebacterium Diphtheriae* (Diphtheria Bacillus)

a. CHARACTERISTICS. These bacilli are slender rods, straight or slightly curved, of medium size; they often lie at various angles to one another, forming V or Y shapes. The organisms are generally not uniform in thickness, exhibiting rounded, pointed, or swollen ends or enlargements along the length of the cell. They usually stain unevenly, showing barred and granular large forms and solid-staining short forms; they are gram-positive, nonacid-fast, and nonmotile. Growth readily occurs at 35° to 36° C. on enriched agar media as circular, smooth, moist, grayish to creamy-white colonies. Some strains produce narrow zones of hemolysis on blood agar. The diphtheria bacillus is pathogenic for man and guinea pigs.

b. **HOST RELATION.** Diphtheria bacilli set up a local infection, usually in the throat. The lesion, however, may also appear anywhere in the upper respiratory tract, or in the esophagus, perineum, or anus, or occasionally as a wound or skin infection. The disease is a result of absorption of an exotoxin produced by the organism in the local lesion. Bacterial invasion of the blood stream ordinarily does not occur. Susceptibility to diphtheria may be determined by the injection of a small amount of the toxin intradermally (Schick test).

c. **DIAGNOSTIC CRITERIA.**

- (1) *General.* These bacilli are characterized by their shape, size, irregular staining, and V or Y arrangements as seen in a smear from the lesion or in one from Loeffler's medium when stained by Loeffler's methylene blue or the modified Albert stain. Since tellurite is reduced, colonies on tellurite medium appear gray to black, depending on the type. Thus, tellurite medium aids in selecting colonies. This reaction is not specific, however, since several other organisms also reduce tellurite. The diphtheria bacillus produces effects in guinea pigs neutralizable by specific antitoxin (see below).
- (2) *Collection of specimens.* A cotton swab is applied to the involved area (throat, nose, or wound) or to the membrane or exudate from that area, with care to gather a considerable amount of the exudate on the swab and with caution not to contaminate the swab by touching it to the tongue or other noninvolved areas. Use this swab for immediate inoculation of appropriate media (see below).
- (3) *Microscopic examination of direct spread.* The swab may be streaked on a slide, fixed by heating, then stained by Loeffler's or a modified Albert's method (par. 248) and examined. It should be strongly emphasized, however, that a negative finding cannot be given value and should not delay treatment in the presence of symptoms. The finding of typical organisms is merely presumptive. Diagnosis cannot be confirmed in the absence of culture and virulence tests. The diphtheria organism usually will be mixed with many others. The presence of Vincent's organisms and diphtheroids should be noted on the report if found.
- (4) *Culture.*
 - (a) Loeffler's serum slants have been used for many years although evidence for the inadequacy of this medium has accumulated. The so-called "typical" morphology

of the diphtheria bacillus is based on stained smears taken from Loeffler's medium. After 18 to 24 hours' incubation at 35° C., growth is examined by making a smear from a broad needle drag along the surface of a slant and staining it as indicated above. The growth also is subcultured to tellurite blood or serum agar and to blood agar for pure colony isolation. If diphtheria-like bacilli are found and the culture is from a suspected case, a presumptive diagnosis should be made at once. If the culture is from a suspected carrier, diphtheria-like bacilli should be further identified by fermentation and virulence tests before reporting.

- (b) The value and limitation of tellurite agar are indicated in (1) above. Many believe it is a more satisfactory medium than Loeffler's for direct isolation, that its use in this way increases the chances of isolating the diphtheria bacillus.
- (c) The blood agar plate provides information on the general bacterial flora, particularly the presence of streptococci, and gives opportunity for notation of colony form of diphtheria-like bacilli.
- (d) An optional, rapid method is to apply a sterile serum-swab to the throat of the patient, return it to the tube, and incubate for a few hours. Transfers are made to other media, and the culture is examined microscopically by gently rolling the swab on a slide to form a thin film. (Serum swabs are prepared by placing sterile swabs in a few milliliters of sterile horse, bovine or rabbit serum. Two hundredths (0.02) percent potassium tellurite may be added to attain blackening from growth of the diphtheria bacilli.)

385. Identification of Diphtheria Bacilli

a. TYPES OF DIPHTHERIA BACILLI. Differences in appearance of colonies and in other characteristics are noted among toxin-producing diphtheria bacilli. Thus the names *gravis*, *mitis*, *intermedius*, and *minimus* have arisen. The significance of determining such "types" is questionable in diagnosis although such distinctions may have epidemiologic significance.

b. DIFFERENTIATION FROM SIMILAR ORGANISMS.

- (1) The diphtheroid bacilli comprise a large group of ill-defined organisms given this general name because of their morphological resemblance to the diphtheria ba-

Table XXXVI. *Diphtheria and Diptheroid Organisms*

Species	Fermentation tests		Hemolysis	Oxygen requirements	Virulence for guinea pigs or chicks
	Dextrose	Sucrose			
<i>C. diphtheriae</i>	+	— (few +)	+ or —	Aerobic and facultative.	+
<i>C. pseudodiphtheriticum</i> (<i>hofmanni</i>).	—	—	—	Aerobic and facultative.	—
<i>C. xerosis</i>	+	+	—	Aerobic and facultative.	—
<i>C. hemolyticum</i> (<i>pyogenes</i> and <i>ovis</i> ?).	+	— or +	+	Aerobic and facultative.	+
<i>C. ulcerans</i>	+	— or +	?	Aerobic and facultative.	+
<i>C. acnes</i>	+	— or +	?	Microaerophilic.....	—
<i>C. typhi</i>	+	—	?	Anaerobic.....	—

cillus. Many are common saprophytes of the throat, skin, and other body areas, and are so ubiquitous that any association of them with specific diseases must be questioned. They are definitely distinguished from virulent diphtheria bacilli by means of virulence-neutralization tests, although most of these are completely avirulent for guinea pigs. Some do not ferment dextrose, whereas diphtheria bacilli ferment this sugar. Some ferment sucrose, a characteristic not usually seen in diphtheria bacilli although sucrose-fermenting forms have been found in recent years. A list of commonly found diptheriods is given in table XXXVI. Classification of the diptheroids is unsatisfactory at present.

- (2) Diptheroids which produce reactions not neutralized by diphtheria antitoxin, in the skin of guinea pigs, have been described with increasing frequency in recent years. Wide use of the Fraser-Weld virulence and neutralization test (see below) probably is responsible for detecting these organisms. Many have been found in human skin lesions where the isolation of *C. diphtheriae* was anticipated. Some of these produce a soluble hemolysin and have been termed *C. hemolyticum*. They produce edema with central induration in the guinea pig skin and resemble organisms previously described as pathogens for animals (see table XXXVI). Others, termed *C. ulcerans*, necrotize the injected area of guinea pig skin.

c. VIRULENCE AND NEUTRALIZATION TEST. The only certain method of identifying *C. diphtheriae* is to demonstrate toxicity in guinea pigs or chicks which is prevented by injecting specific antitoxin. This test is especially employed in carrier studies.

- (1) *Subcutaneous methods.* Inject, subcutaneously, into two 250-gm guinea pigs, 2 ml of a 48-hour broth culture or 1 ml of a suspension of growth suspended in 2.5 ml of broth (not saline solution). One of the guinea pigs should have received, 24 hours previously, an intraperitoneal injection of 250 to 500 units of diphtheria antitoxin, and serves as the control animal. If the organism is a virulent strain of diphtheria bacillus, the unprotected animal dies within 4 days. Post-mortem examination will show gelatinous edema around the site of inoculation and may also show enlarged hemorrhagic adrenal glands. The control animal should survive. Healthy young chicks (5 to 20 days old) may also be used for the test. About 1 ml of a 48-hour broth culture is injected into the subcutaneous areolar tissue over the insertion of the wing. The control chick should have received 250 to 400 units of antitoxin injected into the same area about 1 hour before the culture. If the culture is virulent the unprotected chick dies in 1 to 3 days.
- (2) *Fraser-Weld Intracutaneous Method.*⁴ This method employs a single guinea pig (or rabbit), eliminating the need for an additional animal for antitoxin control. Also, several cultures may be tested at the same time on the same animal. Clip or shave the hair from the back of the animal. A heavy, even suspension of microorganisms is made by adding an appropriate amount, 2 to 3 ml, of meat infusion broth to an 18 hour growth on a slope of tellurite blood agar or Loeffler's serum. With a syringe graduated in 0.1 ml and needle of 26 gauge, 0.1 ml of the suspension is injected *into* the skin of the back of a rabbit or guinea pig. The suspension may be retained in the syringe and stored in the cold for 4 to 5 hours, at which time it is again similarly injected into the animal after the injection of 500 to 1,000 units of antitoxin intraperitoneally in the case of the guinea pig or intravenously in the rabbit. The second injection of organisms may be made immediately after the intravenous injection of antitoxin, but 30 minutes should elapse in the intraperitoneally injected animal. Lesions reach their height in the guinea pig after 48 hours and after 72 hours in the rabbit. The lesion produced by the *C. diphtheriae* shows a central

⁴Diagnostic Procedures and Reagents, Amer. Pub. Health Assoc., 2d Ed., 1945, p. 282.

necrotic area 2 to 5 mm in diameter surrounded by a zone of redness 10 to 15 mm in diameter. Lesions in the rabbit are more intense than in the guinea pig but take longer to develop. The area injected with *C. diphtheriae* after the antitoxin is given produces only a pinkish papule about 5 mm in diameter. If the culture contains *C. hemolyticum*, *C. ulcerans*, virulent streptococci or staphylococci, lesions will appear in the areas inoculated before and after the antitoxin injection. Nonvirulent diphtheroids will produce no (or mild) reaction both before and after the antitoxin is given. If available, a known toxic strain of *C. diphtheriae* should be injected for a positive control.

- (3) *Specific in vitro test for diphtheria toxin production.* A simple procedure for detecting the ability of a culture to produce specific diphtheria toxin avoids the use of animals. A strip of filter paper containing specific antitoxin is embedded in a special agar. The culture to be tested is streaked across the strip. Lines of flocculation occur at areas of optimum concentration of toxin and antitoxin if the organism produces diphtheria toxin. Only certain lots of antitoxin may be used. (Amer. Jour. Pub. Health 40:704, 1950.)

386. *Mycobacterium Tuberculosis* (Tubercle Bacillus)

a. CHARACTERISTICS. Tubercle bacilli are small, slender, straight, or slightly curved rods with rounded ends, occurring singly or in clumps. They are stained with difficulty, but when once stained they resist decolorization with acid-alcohol (acid-fast). They are gram-positive and nonmotile. The organisms may stain unevenly, showing granular, beaded, or banded forms. Growth is aerobic, occurring slowly and producing wrinkled colonies suggestive of cake crumbs on both solid and liquid media. Special media are used, which incorporate glycerin, coagulated egg, or other enrichment material. Growth under the most favorable conditions develops only after a minimum of 10 days' to 2 weeks' incubation at 37° C. and may require 4 to 6 weeks. Colonies are rough, irregular, wrinkled, and dry. A yellow pigment is produced that varies in intensity with the age of the culture, becoming brownish in old cultures.

b. HOST RELATION. There are four types of tubercle bacilli—human, bovine, avian, and strains that infect cold-blooded animals. Of these types only the human and bovine strains are of medical importance. The incidence of bovine tuberculosis in this country

has fallen to a low level, but where unpasteurized milk from infected cows is widely used there is a high incidence of lymph node and intestinal infections among children. The human and bovine types may be differentiated by morphological and cultural characteristics and by their specific pathogenicity for guinea pigs and rabbits. The human strain is a long, slender rod as compared with the shorter, thicker organism encountered in tuberculous cattle. The bovine type is cultivated with greater difficulty than is the human strain, and is more pathogenic for rabbits. Almost any tissue in the body is susceptible to invasion by the tubercle bacillus, but it is most commonly encountered in the lungs; it is also found in lymph nodes, the gastrointestinal and genitourinary tracts, the meninges, cold abscesses, etc. The usual sources for diagnosis are sputum, gastric washings, urine, pus, cerebrospinal fluid, and sometimes feces. Many saprophytic species of acid-fast bacilli, existing in nature and commonly found in soil and dairy products, have been described. *M. smegmatis*, occasionally found in voided urine, may be mistaken for the tubercle bacillus.

c. SPECIAL METHODS.

- (1) *Microscopic examination.* A presumptive diagnosis can be made by applying an acid-fast stain, such as Ziehl-Neelsen carbol-fuchsin, to a slide spread of selected (caseous) fragments of the sputum. The red, acid-fast bacilli are readily noted in contrast to the blue counterstaining of all other bacteria, cells, and debris. Bright illumination is required. Stained spreads may also be from the centrifuged sediment of urine or spinal fluid. A small film of gelatin or sterile egg albumin on the slide will help prevent the sediment being washed off during the staining process. The demonstration of the organism microscopically is presumptive evidence of infection, and in many cases is sufficient.
- (2) *Concentration methods.* Urine samples or other body fluids may be concentrated by centrifuging, after adding sodium oxalate (see par. 232). If sputum or gastric washings contain too few tubercle bacilli to be found by the direct smear, the organisms may be concentrated by digesting the mucus with Hank's solution, sodium hydroxide, trisodium phosphate or clorox. Wherever possible, sediments obtained with any of these reagents (except clorox) should be cultured and/or inoculated into guinea pigs. The tubercle bacilli remain viable after correct use of any of these digestants except clorox. Gastric washings should receive prompt attention for

successful culture or guinea pig inoculation. (Organisms may become nonviable if the specimen is not treated soon after collection.) The samples may be placed directly in trisodium phosphate (see below) or neutralized with 4 percent sodium hydroxide.

- (a) *Hank's solution*. This material contains sodium hydroxide for digestion and potassium alum which forms a floc and thereby increases the efficiency of centrifugation. Directions for its use are given in detail in (3) below.
- (b) *Sodium hydroxide*. Add to the specimen an equal volume of 4 percent sodium hydroxide containing 2 ml of a 0.2 percent solution of phenol red indicator per 100 ml of sodium hydroxide. (The preparation of phenol red indicator is given in paragraph 271e(3) (a).) Shake preferably in a mechanical shaker, for 10 minutes to homogenize the specimen. Centrifuge for 30 minutes at 3,000 rpm. Discard all but a drop or two of the supernatant fluid. Add hydrochloric acid solution (see (3) below) drop by drop until the color changes to yellow. Then back-titrate with a drop or two of the 4 percent sodium hydroxide until the color barely changes to pink. Although less efficient in removing all the bacilli from suspension, this method is frequently preferred to the flocculation method (3) because it produces less extraneous sediment and the sediment is not irritating to the guinea pig tissues.
- (c) *Trisodium phosphate*. This material is recommended for samples which cannot be digested immediately and for specimens which are to be mailed. After exposure to trisodium phosphate for several days, the specimen is digested and contaminants are killed. Tubercle bacilli remain viable after a week's exposure. Mix the specimens with an equal volume of 23 percent trisodium phosphate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) in a screw cap bottle. Mailed specimens are usually digested by the time they reach the laboratory. If not, allow to stand until digestion is evident. Then centrifuge for 30 minutes at 3,000 rpm. Discard the supernatant, wash the sediment with 10 to 15 ml of sterile saline and centrifuge as before. Use the sediment for slide, culture, or guinea pig inoculation.
- (d) *Clorox*. This solution digests rapidly (3 to 5 minutes) and is well adapted to the mass handling of specimens to be examined only by slide smear. Equal volumes

of specimen and clorox (approximately 8 to 10 ml of each) are mixed in screw cap bottles. Shake well and allow to stand for 3 to 5 minutes. If not digested, add additional clorox and shake. Then add 8 to 10 ml of a 4 percent aqueous solution of sodium thiosulfate and shake well. Allow to stand for at least 5 minutes then centrifuge at 3,000 rpm for 30 minutes. Discard the supernatant and smear the sediment on a slide with a cotton swab. The tubercle bacilli are killed in the process, hence culture and guinea pig inoculation should not be attempted after clorox digestion.

(3) *Flocculation method.*

(a) *Reagents.* The digesting solution is prepared as follows:

Sodium hydroxide	40 gm
Potassium alum	2 gm
Brom-thymol blue	0.02 gm
Water q.s. ad	1,000 ml

The acid (about 2.5*N*) solution is prepared as follows:

Hydrochloric acid (concentrated)	250 ml
Water, q.s. ad	1,000 ml

(b) *Test.* Mix 3 to 5 ml sputum with 1 to 4 parts of the digesting solution, and shake well. Incubate at 37° C. for 30 minutes for culture or animal inoculation, or to a homogeneous mass for microscopic examination. Adjust to pH 7.0 (bluish green) with the acid solution. Centrifuge at top speed for 15 minutes, and discard the supernatant fluids. Make smears, inoculate isolation media, and inoculate animals with a saline suspension of the sediment.

(c) *Result.* The sodium hydroxide digests the organic matter. Flocculation occurs when acid is added. This precipitate carries into the sediment the organisms, including tubercle bacilli, which are not killed or dissolved by the alkali.

(4) *Animal inoculation.* Concentrate specimens as indicated in (2) and (3) above and suspend the sediment in 1 or 2 ml of sterile physiological saline solution. Inject this material subcutaneously and intramuscularly into the thigh of a young guinea pig (250 gm). Only tuberculin negative guinea pigs should be used. Test by injecting intracutaneously 0.1 ml of a freshly prepared dilution of old tuberculin containing 1 mg of old tuberculin. Read the reaction in 3 days. Old tuberculin is preferable to purified protein derivative for testing guinea pigs. Observe the guinea pigs for a period of 6 to 8 weeks and

autopsy any pigs which die. Pigs which remain alive may be killed after 6 to 8 weeks and autopsied. At autopsy observe for caseous lymph nodes and for tubercles in the liver and spleen. Confirm the diagnosis by demonstrating acid-fast bacilli in smears from the lesions. The period of observation may be shortened by tuberculin testing guinea pigs at the end of 4 weeks. If the test is positive the animal may die; otherwise it should be killed and autopsied. If the test is negative continue the period of observation another 2 to 4 weeks.

- (5) *Culture*. This is a valuable method for demonstrating the presence of tubercle bacilli in specimens containing only a few organisms. Several loopfuls of the sediment in a concentrate, or a tissue fragment, are planted on the surface of at least 5 tubes of Lowenstein-Jensen medium (see par. 272*n*). Obtain this medium from a reference laboratory if facilities for preparation are not available. Screw-capped tubes are preferred. The caps should be loosened weekly after inoculation to admit air. Incubate at 37° C. for 6 weeks and examine weekly for colonies of *M. tuberculosis*. Where possible, animal inoculation should be used in parallel with the inoculation of media, particularly if acid-fast organisms are seen in smears of the original sample. Thus, culture characteristics and pathogenicity will be checked with a minimum expenditure of time. (See (4) above.) Positive cultures should be checked for pathogenicity by inoculating guinea pigs because of the wide distribution of acid-fast saprophytes.

387. *Mycobacterium Leprae* (Leprosy Bacillus)

a. *CHARACTERISTICS*. These are small, slender rods resembling tubercle bacilli, straight, rarely bent or curved, with rounded ends; they are acid-fast and tend to be arranged in packets or bundles, particularly in mononuclear phagocytic cells. They cannot be cultivated, and are not pathogenic for guinea pigs.

b. *HOST RELATION*. They are found in the various lesions of leprosy, except the anaesthetic areas of nerve leprosy. They are especially easy to demonstrate in nasal and skin lesions.

c. *DIAGNOSTIC CRITERIA*. Leprosy bacilli are acid-fast and occur in packets, chiefly within mononuclear phagocytic cells; they are not recoverable in culture or by guinea-pig inoculation.

d. *SPECIAL METHODS*.

- (1) *Collection of specimen*. As the initial lesion of leprosy is

often an ulcer at the junction of the bony and cartilaginous portions of the nasal septum, swabs or scrapings from this or other nasal lesions are spread onto glass slides. In skin lesions, with a sterile safety razor blade, quickly make a small incision through the thickened area and, without removing the blade, depress the upper edge so that a scraping is made of the cut skin from below upward. Prepare slides from this scraped material. A deep, not a surface skin scraping, is desired for this spread.

- (2) *Microscopic examination.* Spreads made as above are fixed, stained by the Ziehl-Neelsen method, and observed for acid-fast bacilli. Leprosy bacilli are more easily decolorized than are tubercle bacilli, therefore, decolorization must not be carried too far.

388. *Actinomyces* (*Streptothrix*, *Nocardia*)

a. **CHARACTERISTICS.** The actinomycetes have characteristics that relate them to both the bacteria and the fungi, and are therefore included in bacteriological and mycological classifications. They grow in the form of a delicate branched mycelium that is gram-positive. On aging the terminal hyphae break up into bacillary "arthrospores," which function as conidia. In old cultures, and sometimes in pus, these arthrospores resemble diphtheroid bacilli and may be mistaken for such. In a stained section of tissue, the *Actinomyces* colony appears as a central mass of tangled gram-positive filaments, the peripheral hyphae extending radially into the surrounding tissue; hence the name "ray fungus." Especially in the anaerobic species, the ends of the peripheral hyphae are encased in club-shaped sheaths that are gram-negative. When these colonies are found free in pus they are slightly yellow and are the so-called "sulfur granules."

b. **HOST RELATION.** The genus *Actinomyces* comprises 60 or more species, only a few of which are animal pathogens, the others being plant parasites or saprophytes of soil origin. The species, pathogenic for animals, are generally anaerobic, whereas the plant parasites and saprophytes are aerobic. Anaerobic species are frequently harbored in tonsillar crypts and between the teeth and gums. It has been suggested that these may be the sources of systemic infection; the *Actinomyces* are implanted by mechanical injury, by aspiration into the lungs, or by ingestion. The primary lesion may be oral, pulmonary, or intestinal (especially in the appendix), but frequently is not determined. Metastases occur, producing nodular abscesses in various parts of the body. Aerobic

species also have been found in human infections, described as meningitis, pseudotuberculosis, madura foot, etc., the latter often caused by other fungi. Some authorities maintain that the anaerobic actinomycetes of human origin, including *A. hominis*, are identical with *A. bovis*. Of the aerobic species, *A. asteroides* is acid-fast, and *A. madurae*, nonacid-fast.

c. DIAGNOSTIC CRITERIA.

- (1) *Microscopic examination.* If characteristic serous pus is available, look for sulfur granules, crush one of them gently under a cover slip on a slide and, with the microscope, look for the ray-fungus appearance.
- (2) *Cultivation.* It is difficult to cultivate and isolate the slow-growing actinomycetes from material containing bacteria. In pus aspirated from unopened abscesses, however, pure cultures of *Actinomyces* may be obtained. Suitable media are serum (or blood) agar or broth, which are incubated anaerobically or aerobically as may be required. Brewer's fluid thioglycollate medium, enriched with a few drops of serum, is also an excellent medium in which small colonies resembling the sulfur granules are formed. (See also par. 399c for methods employed in culturing the *Actinomyces*.)

389. Spirochetes

a. CHARACTERISTICS. Spirochetes are slender, undulating, corkscrew-like, flexible, filamentous organisms. They have short or long spirals with the twists in three dimensions. The number, depth, relative length, and sharpness of angle of the spirals are of diagnostic importance, although somewhat variable. They are motile by sinuous, rotating movement of the body, not by flagella as in the case of bacteria. They stain with difficulty by ordinary stains although some (*genus Borrelia*) stain readily. The polychrome methylene blue stains of Wright and Giemsa are generally used. The silver-impregnation method is applicable to the more resistant forms, Fontana stain for spreads, and Levaditi stain for tissues. They are most readily demonstrable in the fresh state by dark field illumination. Cultivation is difficult, and generally not practicable. Animal inoculation is of value in the diagnosis of a few spirochetal infections.

b. HABITAT. Spirochetes are ubiquitous, occurring in nature in soil, water, and decaying organic materials, and on and in the bodies of man, animals, and plants. Some are saprophytes, others are commensals. A few are pathogenic, causing such severe diseases as syphilis, yaws, relapsing fever, and Weil's disease.

390. *Borrelia Recurrentis* (Relapsing Fever Spirochete)

a. CHARACTERISTICS. These are spirochetes having large, wavy, inconstant spirals, usually about five. When seen under dark-field illumination, the organisms are very active, in length are several times the diameter of an erythrocyte, and rapidly progress in either direction, disturbing the red cells by their motion. The *Borrelia* stain readily and uniformly by polychrome stains (Wright or Giemsa) and by simple stains. When inoculated into mice and rats, they cause periodic spirochetemia without demonstrable clinical symptoms. Cultivation is not practical.

b. HABITAT. The cause of relapsing fever, the spirochetes, are found in blood and tissues of patients suffering from relapsing fever and in the body and intestinal contents of infected vectors (ticks and lice). *B. recurrentis* applies to the spirochete of European relapsing fever. A number of other species exist. Names have been given for the spirochetes of the United States and Mexico (*B. turicata*), Central and South America (*B. venezuelensis*), and others, differentiation of which is based only on specific immunological reactions. Some lower animals may serve as reservoirs of infection—in the United States, the armadillo and the opossum.

c. IDENTIFICATION.

- (1) Fresh or citrated blood, taken during a febrile paroxysm, and diluted with saline is examined by the dark-field method for spirochetes with the characteristic motility and morphology. A slide spread, stained by the Giemsa method or with dilute carbolfuchsin, should also be examined. The organisms, however, may be much distorted, the spirals often obliterated, so that the characteristic morphology cannot be found. The spirochetes may sometimes be detected and the diagnosis suggested in a routine Wright's stain for a differential blood count.
- (2) A white mouse or rat should be injected intraperitoneally with 0.2 to 0.5 ml of blood, and fresh tail blood examined for spirochetes from the second to fourteenth day.

391. Fusospirochetal Disease (Vincent's Angina)

Vincent's angina is an inflammatory lesion in the mouth, pharynx, or throat, most often affecting the gum margins and tonsils. An acute inflammation may lead to the formation of a pseudo-membrane, suggesting that of diphtheria. Later there are punched-out ulcers, suggestive of syphilis. The disease is localized, generally mild and with minimal systemic disturbances. Although the primary etiology of this disease is not known, the two micro-

organisms described in the following two paragraphs are almost always found together, in great numbers, in smears from the lesions, the two organisms apparently living in symbiosis. They are rarely present alone, being usually accompanied by other micro-organisms, such as staphylococci, streptococci, and even diphtheria bacilli, the last finding being more significant than the Vincent organisms.

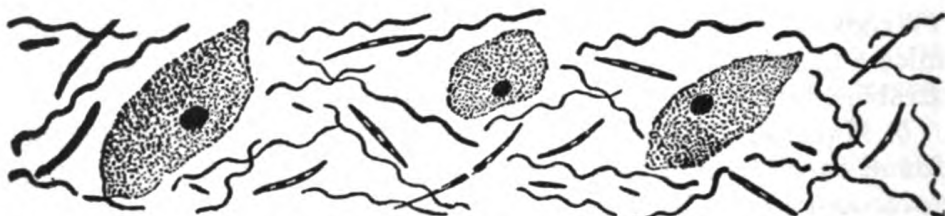


Figure 28. Vincent's angina. (Note presence of *Borrelia vincentii*, fusiform bacilli, and epithelial cells.)

392. *Fusobacterium Plauti-vincenti* (Fusiform Bacillus of Vincent)

This is a large bacillus, thick in the middle and tapering toward the ends to blunt or sharp points. It is readily stained by Loeffler's methylene blue, carbol-fuchsin, or the Giemsa stain, with characteristic inequality in the intensity of the stain, being more deeply stained near the end, often with alternating bands of stained and unstained areas in the central body, not unlike the bands of diphtheria bacilli.

393. *Borrelia Vincentii*

This is a spirochete somewhat like that of relapsing fever, and is longer than the fusiform bacillus; it is made up of variable numbers of undulations, shallow and irregular in their curvatures, unlike the more regularly steep waves of *Treponema pallidum*. They stain more evenly and less distinctly than the fusiform bacilli. To identify make slide spreads from the ulcerative lesions, fix in the flame, stain deeply with dilute carbol-fuchsin, crystal violet, or Wright's stain, and examine for fusiform bacilli and spirochetes. Positive results will be evidenced by finding great numbers of both fusiform bacilli and spirochetes. A few forms of either type are not significant.

394. *Treponema Pallidum* (Spirochete of Syphilis)

a. CHARACTERISTICS. This is a delicate spirochete coiled in 8 to 14 regular, rigid, sharp spirals that are equal or greater in depth than in length, with acute rather than obtuse angles. As

seen under dark-field illumination, it appears as a highly refractile, long, slender, spiral, silvery form with serpentine, corkscrew-like movement. It is motile, but does not progress rapidly or far, the motion being rotational with undulations. The spirochetes are made visible most effectively by dark-field illumination. They are difficult to stain with aniline dyes other than Giemsa stain; the body stains pink by the Giemsa method and black by the silver impregnation method (Fontana stain in spreads, Levaditi stain in tissues.)

b. HOST RELATION. Naturally occurring only in human beings, it causes the disease syphilis, with protean manifestations. It is transmitted only by direct contact, generally through sexual intercourse, occasionally through intimate contact of other mucous membrane or skin sites. Syphilis, one of the most prevalent and important of all infectious diseases, usually progresses through a number of irregular and varied stages. During the incubation period, which lasts 4 to 6 weeks, the spirochetes cannot be demonstrated. In the primary stage, a chancre appears at the site of infection; this starts as a papule, enlarges, becomes hardened, and then ulcerates, forming an ulcer with a firm base and hard edge in the typical form. Atypical penile lesions frequently occur, especially if secondarily infected or coexistent with chancroid. Spirochetes can be found in the fluid expressed from a chancre. The spirochetes will not necessarily be on the surface, but rather in the deep tissues and in the serum exuding from a scarified lesion. At this stage they already have become disseminated as a general infection, can be demonstrated in fluid aspirated from regional lymph nodes, but cannot be readily found in the blood or other areas. In the secondary stage, which is characterized by mucous patches, skin rashes, and a variety of superficial lesions, *T. pallidum* can usually be found in material from moist secondary lesions. The *tertiary stage* is characterized by deep, rather than superficial, lesions which involve the viscera, bones, and central nervous and cardiovascular systems. Spirochetes are usually scanty, and not readily demonstrated in these lesions.

c. SPECIAL METHODS. Different procedures are applicable to different lesions and stages.

(1) *Dark-field examination.*

- (a) The lesion is cleansed of surface crust, detritus, pus, and surface organisms with gauze or a cotton applicator. If the lesion has been treated with a germicidal agent, examination is deferred until all germicide has been removed and the lesion has had a saline pack applied to it for a day or two.

- (b) Primary lesions are then mildly traumatized to provoke an exudation of serum, by gently rolling the lesions between the gloved finger and thumb or by rubbing its surface with a dry cotton applicator. Hemorrhage should be avoided, although a few erythrocytes or pus cells are desirable to aid in obtaining a proper focus. Secondary lesions are merely cleansed and abraded.
 - (c) Fresh preparations for microscopic examination may be made from accessible lesions by merely touching the slide to tissue juice and immediately placing the cover glass over this drop. Vaseline placed around the edge will prevent drying. If the lesion is less accessible, the fluid may be collected in a capillary pipette and placed on the slide.
 - (d) Examine immediately with the dark-field microscope for the characteristic morphology and motility of *Tr. pallidum*. Exercise caution not to misinterpret observations. There are many saprophytic spirochetes that are easily distinguished. There are, however, a few spirochetes, especially in the mouth, that are extremely difficult to differentiate. Indeed, a diagnosis of syphilis based solely on the dark-field examination of material from the oral cavity is believed to be untenable by most clinicians. "Artifact spirochetes" are sometimes mistaken for spirochetes by those unfamiliar with the appearance of blood, pus, and cultures under dark-field illumination, and wavy filamentous structures may actually simulate spirochetes.
 - (e) Report findings with qualifying data, such as notation of location of lesion examined, the occurrence of conditions making examination unrepresentative, etc.
- (2) *Delayed dark-field method.* When facilities for dark-field examination are not locally available, or when local examiners desire confirmation of their own findings by a consultant, lesion fluids may be forwarded to a distant laboratory for examination. The tissue fluid from a suspected lesion is allowed to flow into a capillary tube about 8 cm long and 1 mm in diameter. The two ends of this tube are sealed by pressing into a soft paraffin-vaseline mixture (50 percent of each) and then forwarded for dark-field examination. At the examining laboratory the serum may be transferred to slide by pressing one end of the capillary tube into a paraffin-vaseline mixture

until the plug in the opposite end is forced out. Such material must be examined within a few days.

- (3) *Nigrosine method.* This is not strictly a staining method for it leaves the unstained spirochete in a black field. A loopful of the fresh tissue fluid is mixed with a loopful of 5 percent aqueous solution of nigrosine, containing 0.5 percent formalin as a preservative. (See par. 253.) This mixture is spread on a glass slide, dried, and examined by ordinary illumination with an oil-immersion objective. A remote examination may be made by forwarding an air-dried drop of the exudate on a slide. The laboratory adds a loopful of water to this to dissolve the exudate and proceeds with the nigrosine preparation. Results are far inferior to the dark-field method, for motility is absent and the spirochetes, by distortion, have lost much of their characteristic morphology.
- (4) *India-ink method.* Like the nigrosine method, a drop of material is mixed with a drop of drawing ink and the mixture spread on a slide. (See par. 249.) When dry, it is examined for white spirals against a dark background.
- (5) *Stained-spread examination.* Spreads may be stained by the Giemsa method. (See par. 464.)
- (6) *Serological tests on blood serum and spinal fluid.* These are applicable to the later stages of syphilis. It is customary to subject all patients with venereal disease, even after repeated, negative dark-field examinations, to follow-up blood tests for several months.

395. *Leptospira Icterohaemorrhagiae* (Organism of Weil's Disease, Infectious Jaundice)

a. CHARACTERISTICS. The spirochetes show many coils, so fine as to be difficult to detect. One or both ends may be bent into a hook. Rapid, spinning motion with intermittent active lashings is characteristic. They are difficult to stain, being stained reddish by the Giemsa method, cultivation is possible only by special methods. Diagnosis can be made by the injection of blood or urine into a guinea pig.

b. HOST RELATION. The blood and kidneys of infected wild rats and the blood, urine, kidneys, and biliary tract of patients with infectious jaundice (Weil's disease) harbor the organisms.

c. IDENTIFICATION.

- (1) *Guinea-pig inoculations.* Inject 3 to 5 ml of fresh blood, fresh urine sediment, or tissue suspension, intraperitoneally into a white guinea pig. Observe it daily for

fever, for jaundice of the ears, eyes, and about the genitalia, and for *Leptospira* in the blood (usually found after the fourth day). After the animal dies, large numbers of spirochetes can be demonstrated in emulsions of the liver, kidneys, and adrenal glands.

- (2) *Dark-field examination.* Tissue emulsions and occasionally urinary or biliary sediment, may be examined under the dark-field microscope for motile *Leptospira*.
- (3) *Stained spreads and cultures.* These have limited application.

d. **SEROLOGICAL REACTIONS.** Special agglutination and complement fixation tests are valuable in diagnosing leptospiral infection, but the technics and reagents are not yet generally available. Serum and clinical abstracts should be forwarded to the Army Medical Service Graduate School.

Section II. MYCOLOGY

396. General

The plant kingdom is divided into four large groups: *Spermatophyta* (seed plants), *Pteridophyta* (fern plants), *Bryophyta* (moss plants), and *Thallophyta* (simple vegetative structures not differentiated into roots, stems or leaves).

a. There are two subgroups of *Thallophyta*, *the algae*, containing chlorophyll, and *the fungi*, simple plant organisms containing no chlorophyll, and existing as saprophytes or parasites on organic material. Single-cell types of fungi, such as the common budding yeasts (for example, *Saccharomyces cerevisiae*), grow much as do bacteria except for their method of multiplication (by budding, not by fission). Each individual cell combines the functions of nutrition and reproduction. Other types of fungi, the molds, are made up of many cells, usually cylindrical or tubular and forming *hyphae*. The branched hyphae together make up a *mycelium*. Specialized branches of the hyphae bear spores, which vary greatly in size and shape. The spores and the structures that bear them are characteristic for each species of fungus.

b. Fungi are widespread in nature. The laboratory worker must constantly remember that saprophytic species of fungi frequently contaminate lesions and cultures. Some fungi, such as those that give flavor to cheese and cause bread to rise, are of commercial importance. Relatively few species are capable of producing disease in man.

(2) *Yeastlike:*

<i>Cryptococcus neoformans</i> (<i>Torula histolytica</i>)	Cryptococcosis
<i>Candida (Monilia) albicans</i>	Moniliasis

(3) *Moldlike:*

<i>Blastomyces dermatitidis</i>	North American blastomycosis
<i>Blastomyces (Paracoccidioides) braziliensis</i>	South American blastomycosis
<i>Histoplasma capsulatum</i>	Histoplasmosis
<i>Coccidioides immitis</i>	Coccidioidomycosis
<i>Sporotrichum schenckii</i>	Sporotrichosis
<i>Hormodendrum pedrosoi</i>	Chromoblastomycosis
<i>Hormodendrum compactum</i>	Chromoblastomycosis
<i>Phialophora verrucosa</i>	Chromoblastomycosis
Species of <i>Madurella</i> , <i>Monosporium</i> , <i>Indiella</i> , etc.	Mycetoma and Madura- mycosis, Madura foot

b. **SUPERFICIAL MYCOSES.** The fungi causing superficial mycoses are localized in the skin, hair, and nails (sources of material for laboratory examination). They do not penetrate the deeper tissues, do not metastasize, and are not life-endangering. They may be divided into three groups:

- (1) *Microsporum* attacks skin and hair of the scalp and face; infrequently other areas of the body are involved; does not attack nail tissue.
- (2) *Trichophyton* attacks skin, nails and hair; not confined to any one body area.
- (3) *Epidermophyton* attacks nails and skin. Although not confined to the lower extremities it has a particular affinity for the inner aspect of the thighs; hair not involved.

399. Methods

a. **COLLECTION OF SPECIMENS.** To secure material for examination and culture the following instruments should be kept at hand in a sterile condition:

Dull scalpel.

Sharp, pointed scissors.

Small syringe with 19- or 20-gauge needle.

Forceps (preferably small).

Single-edged razor blade.

Bacteriologic loop (heavy wire).

Two stiff, sharp or pointed wires (for tenacious materials and cultures).

Sterile Petri dishes.

- (1) Select active, infected areas; dried and apparently inactive lesions, however, may be best for *microscopic demonstration* of dermatophytes.
- (2) Cleanse the affected part with 70 percent ethyl alcohol.
- (3) Remove materials aseptically.
- (4) Collect hairs, skin, or nail scrapings in sterile Petri dishes.
- (5) Aspirate pus from a deep part of the lesion (exposed pus contains numerous saprophytic species).
- (6) Collect sputum in sterile containers, having the patient first use a mouth-wash. If a systemic mycosis is suspected, a bronchoscopic specimen should be obtained to eliminate fungi present in the mouth as saprophytes. For such organisms as *H. capsulatum*, *B. dermatitidis* and *C. immitis*, a series of gastric washings also should be cultured.
- (7) Deep portions of nails are not likely to contain saprophytes. Scrape well below the surface. Use small flakes for direct examination and culture.

b. DIRECT EXAMINATION.

- (1) *Hair, skin, and nails.*
 - (a) Place a drop of 10 percent potassium hydroxide on one end of a clean glass slide.
 - (b) Add a small flake of the tissue to be examined.
 - (c) Cover with a cover glass.
 - (d) Pass the slide through a low flame several times (the pilot light will suffice).
 - (e) Examine the slide under the low-power and the high-power lens for the presence of filaments and spores. Fungi resist the digestive action of the hydroxide, whereas the tissue elements disappear. Some workers leave the preparation standing for several hours, but repeated gentle heatings facilitate the digestive action to such an extent that examinations can be made almost immediately. Avoid mistaking artefacts for yeast-like organisms and hyphae. The inexperienced worker should familiarize himself with the microscopic appearance of uninfected tissue.
- (2) *Sputum and pus.* No alkali is usually needed for direct examination. Examine a drop under the low-power and the high-power lens for hyphae, yeastlike cells (budding), spherules containing endospores, etc. If no organisms are seen, prepare a second slide by adding to the

drop of pus a drop of clearing fluid (aqueous solution containing 5 percent potassium hydroxide and 25 percent glycerin) and allow it to stand overnight. Examine the following day for the structures enumerated above. Artefacts resembling hyphae and budding cells frequently develop in pus and sputum mixed with hydroxide.

- (3) *Spinal fluid*. Centrifuge the specimen. Examine a wet preparation of the sediment for budding cells. Make a second preparation by mixing a loopful of india ink with a loopful of the sediment on a glass slide; this should be done quickly so that a cover glass can be placed over the mixture before it dries. This method is excellent for capsule demonstration.
- (4) *Vaginal discharge*. Wash the swab containing the material in warm physiological saline solution. Centrifuge. Make a wet preparation of the sediment and examine it for budding cells and rudimentary hyphae.

c. CULTURE.

- (1) *General*. The addition of 25 units of penicillin and 40 units of streptomycin each per ml of medium, adequately inhibits the growth of most gram-positive and gram-negative bacterial organisms, but has no inhibitory effect upon the growth of any of the pathogenic fungus species except the *Actinomyces*. These antibiotics, therefore, cannot be incorporated in media employed in the isolation of agents of suspected actinomycosis, but for all others it is a recommended procedure. Media containing the antibiotics can be heavily inoculated, thereby increasing the possibility of isolation.
- (a) Thus, in addition to the specialized media cited below which may not be available in certain of the smaller laboratories, any basic meat infusion agar can be made into an acceptable fungus isolation medium by adding 1 percent glucose and/or blood and the antibiotics.
- (b) Isolation of pathogenic fungus agents is enhanced by use of freshly prepared, moist media. Basic media are stored in 100 ml lots at 5° C. Individual lots are melted as needed, and cooled to 45° to 50° C. The antibiotics, glucose, blood, or other enriching materials are added at this temperature and mixed thoroughly just prior to dispensing the medium in sterile plates or tubes.
- (c) Plates after inoculation are sealed with Parafilm or a similar substance and incubated right side up until the inoculum has been absorbed by the medium. The

depth of medium in either plates or tubes should be sufficient to compensate for the long incubation period required for the isolation of many fungus pathogens. Certain pathogenic isolates may appear as early as 48 hours after inoculation, but for species which are extremely slow growing, an incubation period of from 3 to 6 weeks is required. All inoculated media should be observed for at least 3 weeks before being reported as negative.

- (d) Organisms such as *B. dermatitidis*, *B. brasiliensis*, *H. capsulatum*, and *S. schenckii* can sometimes be cultured in the yeast phase. This requires 37° C. incubation on enriched, moist medium such as (b), (e), or (j) described below. The procedure is not recommended for isolation purposes and should be pursued only if specialized problems require yeast phase cultures of the organisms.
 - (e) *Cryptococcus* species should also be cultured at 37° C., since pathogenic strains usually are the only ones growing at this temperature.
 - (f) Except in these two instances and in culture of *A. bovis*, room temperature incubation results in a greater number of isolations.
- (2) *Selection of media.* One or more of the following media to which penicillin and streptomycin have been added is recommended for isolation. All should be incubated at room temperature.
- (a) Sabouraud's (maltose or dextrose) agar (see par. 272o).
 - (b) Brain heart infusion agar containing 1 percent glucose (see par. 272t).
 - (c) Crystal violet oxgall agar (no penicillin necessary) (see par. 272u).
 - (d) Potato dextrose agar (see par. 272v).
 - (e) Blood agar containing 1 percent glucose.

The above media, except (c), also are satisfactory for the isolation of *Actinomyces* species, if the antibiotics are not added. Suspected actinomycotic materials should be inoculated on parallel sets of media incubated at 37° C. (aerobically for *Nocardia* species, anaerobically for *Actinomyces bovis*). Fluid thioglycollate is also an excellent medium for the study of the latter organism.

The following media without antibiotics should be used for pure culture studies. Incubation is at room temperature unless otherwise noted.

- (f) Sabouraud's agar.
 - (g) Potato dextrose agar.
 - (h) Corn meal agar (*Candida* species).
 - (i) Carrot plugs (yeast species).
 - (j) Dextrose-cystine blood agar (par. 272k) for yeast phase culture of *Blastomyces*, *Histoplasma*, and *Sporotrichum*. Incubate at 37° C.
- (3) *Inoculation of media.* Do not culture materials digested by NaOH or other concentration methods. Pathogenic fungi do not survive them. Centrifugalization of liquid and maceration of tough or tenacious specimens, however, is recommended. Use sterile swabs, instead of bacteriologic loops, for streaking plates.
- (a) Culture specimens of thick sputum and pus, as well as feces, as received. Centrifuge liquid specimens and plate the sediment.
 - (b) Centrifuge specimens of spinal fluid, urine, pleural fluid, and gastric washings and plate the sediment.
 - (c) Grind biopsy specimens in a sterile mortar, using sterile sand if necessary. Dilute suspension with 1 to 2 ml of saline and plate.
 - (d) Culture specimens of blood and sternal marrow as received in 100 ml lots of veal infusion broth containing 1 percent dextrose and the antibiotics.
 - (e) Culture hair, skin, and nails by placing small flakes of skin and nails, or hair roots, at one-half inch intervals over the medium. A moist inoculating wire facilitates handling of specimens.
- (4) *Observation of cultures.*
- (a) *Macroscopic.* Examine plates unopened until colonies are apparent. Once growth is established, remove parafilm to allow further growth to proceed more rapidly. At this time also, transfer colonies to potato dextrose or Sabouraud's agar slants. Incubate transfers at room temperature and observe daily for rapidity of growth, pigmentation, and type of colony.
 - (b) *Microscopic.* Place a drop of mounting fluid (water, salt solution, or lactophenol—the last is preferable) on a clean glass slide. With a stiff, sharp wire remove a fragment of the culture supported intact in a fragment

of the culture medium to the drop of mounting fluid. In some cases it is better to remove a bit of aerial mycelium only, moisten it momentarily in 95 percent ethyl alcohol, and tease it apart in the mounting fluid with two stiff sharp needles. Cover with a vaseline-ringed cover glass, press gently, and examine under the microscope. The lactophenol is prepared as follows:

Phenol (crystals)	20 gm
Lactic acid (syrup)	20 ml
Glycerin	40 ml
Water	20 ml
Cotton blue (C ₄ B Poirrier)	0.05 gm
(may be omitted).	

Mounts prepared in lactophenol will serve as permanent preparations if the edges of the cover slip are sealed with ordinary fingernail polish. Cover slips are not ringed with vaseline for such preparations and care should be exercised to remove all air bubbles before sealing. Additional heating will also serve to drive the stain into the fungus filaments.

(5) *Slide culture* (for studying the details of growth without disturbing the growing fungus).

- (a) *Preparation of slide.* Impregnate a piece of smooth filter paper by dipping it in melted paraffin. When the paraffin is hard, cut the paper into 4- by 25-mm strips. Sterilize a slide by passing it several times through a flame. While the slide is still warm, place two pieces of the paraffined filter paper across it at such a distance that the cover glass will just cover them. Flame a 22-mm square cover glass, and while it is still warm, place it over the paraffined paper strips on the slide. If necessary, seal the cover glass in place by pressing the side of a hot inoculating wire across the edges in contact with the paraffined papers.
- (b) *Inoculation of slide.* Melt a tube of Sabouraud's or other suitable agar, and cool it to about 45° C. Inoculate it with spores, cells, or bits of mycelium of the fungus. With a warm (flamed) sterile capillary pipette allow a drop or two of the inoculated fluid agar to flow under the edge of the cover glass on the slide until the agar occupies about half the space under the cover glass.
- (c) *Incubation of culture.* It is absolutely necessary to prevent drying of the culture medium on the slide during incubation. This may be accomplished by support-

ing the slide across a couple of toothpicks or matches over two or three thicknesses of moist filter paper in a Petri dish, and then inclosing the entire dish in a covered vessel. Incubate at the desired temperature: for fungi causing systemic infections, 37° C. is usually optimum; for dermatophytes, 30° C.; and for saprophytes, room temperature. During incubation the filter paper in the Petri dish *must* be kept moist.

- (d) *Observation.* From time to time the slide culture should be observed under the low-power or the high-power lens of the microscope. Aerial growth appears about midway under the cover glass, where the line of agar meets the air. Deep growth of vegetative mycelium (or cells) appears below this line.

d. ANIMAL INOCULATION.

- (1) Some of the fungi are not pathogenic for laboratory animals, but a few species, especially those causing systemic infections, are sufficiently pathogenic to make animal inoculation a useful aid in laboratory diagnosis. Diagnostic information is obtained by the intraperitoneal, intravenous, or intracerebral inoculation of the suspected material into the animal of choice. A pure culture of the isolated fungus suspended or macerated in sterile physiological saline solution is preferable to original clinical material. The forms of the fungi seen in tissues are usually quite different from the forms seen in cultures.
- (2) To produce dermatophytosis in animals, remove the hair from a large area on the side of the abdomen and abrade the exposed surface with sterile sandpaper or a dull blade. Rub well into the tissue a portion of a macerated slant culture of the fungus in pure culture.
- (3) Observe all animals for a prolonged period for the local and general evidences of infection that are specified in table XXXVII.

400. Identification by Direct Examination

The following key can be used for the identification of genera by the direct examination of clinical material; *to definitely identify the species, culture is essential*:

a. SPORES PRESENT. Hyphae present.

- (1) Spores found in and around hairs; branching hyphae in cutaneous scales.

- (a) Small, round spores, irregularly arranged; usually within a sheath, external (ectothrix) to hair.

Microsporum

- (b) Oval, round or cylindrical spores (3 to 8μ); arranged in chains within hair (endothrix), or both within and external to hair. Endothrix hairs with many air bubbles are suggestive of *Achorion-Trichophyton*

- (c) Large, flattened, thick-walled cells and arthrospores; usually confined to skin of inner thighs, or lower extremities, and not invading hair. *Epidermophyton*

- (2) Budding spores (or cells) often detached from rudimentary mycelium. *Candida (Monilia), Endomyces*

- (3) Septate brown or black bodies in clumps. Multiplies by splitting. *Hormodendrum, Phialophora*

b. SPORES OR BUDDING CELLS PRESENT. Hyphae absent.

(1) *Budding forms.*

- (a) Small, round or oval cells (3 to 7μ).

Cryptococcus (Torula)

- (b) Round or slightly oval cells (10 to 15μ) with highly refractile cell walls; single or multiple budding.

Blastomyces

- (c) Thin-walled oval cell (2 to 2.5μ) with single bud at pointed end; round cells also present; large vacuole and active protoplasmic granules within cell.

Histoplasma

(2) *Nonbudding forms.*

- (a) Double-contoured round bodies (5 to 80μ); mature cells tightly packed with endospores. *Coccidioides*

- (b) Cigar-shaped cells, rarely demonstrated except in experimentally infected animal. *Sporotrichum*

c. SPORES ABSENT. Hyphae absent.

- (1) Typhae with few septations. *Aspergillus, Mucor*

- (2) Yellow ("sulfur") granules with a central portion of interlacing hyphae (0.5 to 0.8μ in diameter) surrounded by a radial arrangement of hyphae, sometimes extending into clublike or bulbous sheaths. Gram-positive.

Actinomyces

- (3) Various colored granules composed of hyphae 1 to 4μ in diameter. *Monosporium, Madurella, etc.*

- (4) Bacteria-like rods and branching filaments (0.8 to 1.0μ). Acid-fast. *Nocardia*

Table XXXVII. Animal Pathogenicity Tests

Organism	Animal	Site	Amount	Time	Findings
<i>Coccidioides* immitis</i>	Guinea pig	Intratesticular	2-3 ml	7-10 days	Double-contoured spherules containing endospores in pus aspirated from testes.
	Mouse	Intraperitoneal	0.5 ml	4-10 days	Spherules containing endospores in intra-abdominal abscesses.
<i>Blastomyces dermatitidis</i>	Mouse	Intraperitoneal	1 ml	2-3 weeks	Generalized infections; tubercular lesions in kidneys, lungs, spleen, etc.; budding cells microscopically.
<i>Sporotrichum schenki</i>	Rat	Intraperitoneal	2-3 ml	Variable	Examine at 2-day intervals for lesions (testes, joints, and cutaneous papules on tail). Cigar-shaped organisms.
	Mouse	Intraperitoneal	2-3 ml	(Hold 4 weeks)	
<i>Candida albicans</i>	Rabbit	Intravenous	2-3 ml	4-10 days	Fatal, with numerous miliary abscesses in kidneys. Budding cells.
<i>Cryptococcus neoformans</i>	Mouse	Intracerebral	0.5 ml	24-48 hours	Examine brain material for budding round or oval cells; make a wet india-ink preparation to demonstrate capsules. Mesenteric lesions.
	Mouse	Intraperitoneal	1 ml	3-4 weeks	
<i>Nocardia (Actinomyces) asteroides</i>	Guinea pig	Intraperitoneal	2-3 ml (heavy suspension)	4-10 days	May be fatal. Failure to inject a massive inoculum will result in a transitory infection. Examine all lesions for acid-fast branching organisms.
	Guinea pig	Intraperitoneal	2-3 ml	Variable	May be fatal; examine spleen and lungs for intracellular budding cells.
<i>Histoplasma capsulatum</i>	Rat	Intraperitoneal	2-3 ml	(Hold 6 weeks)	

* Laboratory infections caused by *Coccidioides* are frequent, and old cultures must be handled with extreme caution. Cultures must be used for inoculum. Clinical material injected directly into an animal do not produce the disease even though parallel cultures are positive.

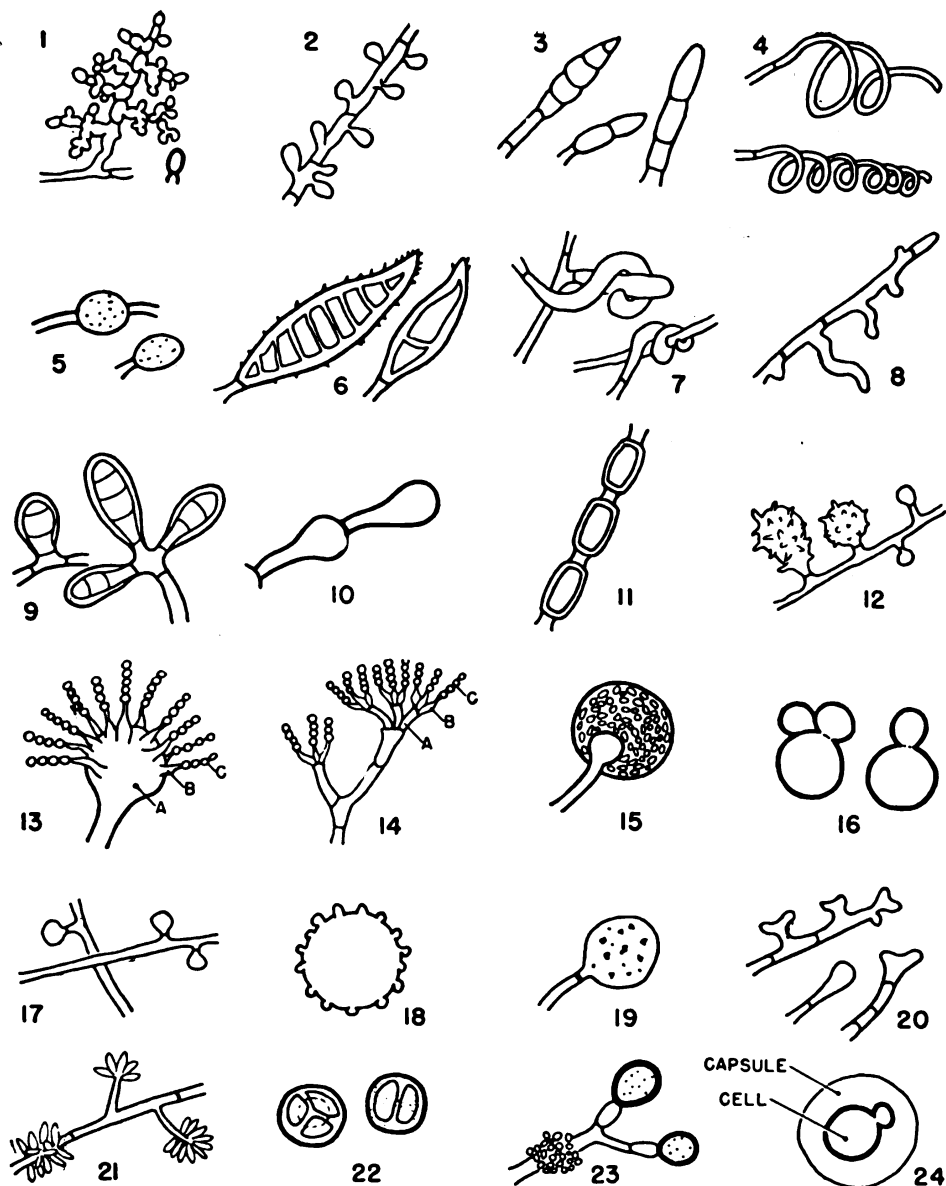


Figure 29. Morphological characteristics of fungi.

401. Identification by Cultural Examination

The following key can be used for the identification of genera, and certain species, by cultural study (the italic numbers in parenthesis refer to the numbered sketches in fig. 29). Morphological characteristics of fungi:

1. Microconidia in clusters (French, *en grappes*).
2. Microconidia along hypha (French, *en thyrses*).
3. Macroconidium (*Trichophyton*) (French, *fuseau*).
4. Spiral.
5. Intercalary chlamydospore.

6. Macroconidium (*Microsporum*).
7. Nodular organ.
8. Pectinate body.
9. Macroconidia (*Epidermophyton*).
10. Racquet mycelium.
11. Arthrospores.
12. "Tuberculate" chlamydospore.
13. Aspergillus.
 - A. Vesicle.
 - B. Sterigmata.
 - C. Conidia.
14. Penicillium.
 - A. Conidiophore.
 - B. Sterigmata.
 - C. Conidia.
15. Sporangium.
16. Single budding cell.
17. Lateral conidia.
18. Multiple budding cell.
19. Cell containing granules.
20. Favic chandeliers.
21. Conidia (*Sporotrichum*).
22. Ascospore.
23. Terminal chlamydospore (*Canida albicans*).
24. Encapsulated cell.

a. Aerial growth; powdery, cottony, feathery, velvety, granular or downy; apparent on solid media after relatively short period of incubation.

- (1) Hyphae present in all cultures. Spores borne on simple fruiting structures (conidiophores).
 - (a) Regularly outlined hyphae. Numerous round or oval microconidia borne in clusters (*en grappe*) (1) and laterally along hyphae (*en thyse*) (2). Macroconidia (3) with blunt or rounded ends produced in some primary cultures. Spirals (4), nodular organs (7), and chlamydospores (5) present in many species. Pigment developed in several species (*T. rubrum*, *T. violaceum*, etc.) *Trichophyton*
 - (b) Hyphae less regular and usually wider than in the above species. Numerous multiseptate (3 to 12 divisions), tapering, sometimes rough-walled macroconidia (6). Few microconidia; more frequently borne *en thyse*. Frequent chlamydospores, racquet mycelium, nodular organs. (7), and pectinate bodies (8) (*M. audouini*). Spirals rare. Yellow pigment formed by some species (*M. lanosum*). *Microsporum*
 - (c) Greenish-yellow to olive-drab pigment. Numerous blunt macroconidia (9) occurring directly on hyphae

- in clusters. No microconidia. Numerous chlamydo-spores. *Epidermophyton*
- (d) White, cottony plaques with moth-eaten appearance. Racquet mycelium (10). Barrelshaped spores in chains, easily dissociated in old cultures (arthrospores) (11). *Coccidioides immitis*
- (e) Septate hyphae from which lateral branches terminate in large spherical or pyroform spores with short fingerlike projections ("tuberculate" chlamydospores) (12). *Histoplasma capsulatum*
- (2) Long, widely septate hyphae. Specialized fruiting structures. Colonies usually green or black. Rapidly growing. (Few, if any, species in this group are pathogenic, but they include the most troublesome laboratory contaminants.)
- (a) Mycelium develops stalks (conidiophores) terminating in large rounded heads (vesicles). From regular fingerlike processes (sterigmata) on the heads, rows of spores (conidia) develop in all directions (13). Some species develop, within perithecia, sacs (asci) (22) containing ascospores. *Aspergillus*
- (b) No vesicle. Sterigmata attached directly to conidiophore (14). *Penicillium*
- (c) Hyphae nonseptate. Spores are contained in large round, thick-walled terminal structures (sporangia) (15). *Mucor, Rhizopus*
- b. Irregularly convoluted growths on solid media, usually without aerial hyphae, or with short aerial hyphae.
- (1) *Slow-growing species.*
- (a) *Short thick hyphae.* Carbohydrates not fermented.
1. Colonies on blood agar incubated at 37° C. friable, yellowish-brown, and tuberclelike. Cells, with highly refractile walls, that bud (16), and frequently develop short rudimentary hyphae. Colonies on Sabouraud's agar (room temperature) covered with short aerial hyphae in central portion and surrounded by moist peripheral zone. Single lateral spores (17) borne on short conidiophores and chlamydospores. *Blastomyces dermatitidis*
2. Colonies on blood agar incubated at 37° C. similar to the above species. Multiple-budding cells (18). Colonies on Sabouraud's agar (room temperature) small, discrete, and covered with scant aerial hyphae. Spherical cells containing granules (19). *Blastomyces braziliensis*

- (b) *Fine branching hyphae*. Colonies frequently colored. Spores when present, small, oval, or pear-shaped.
1. Deep-violet colony. Bizarre hyphae. Few or no spores.
Trichophyton violaceum
 2. Tannish-brown colony, penetrating to crack medium. Numerous structures resembling reindeer horns ("favic chandeliers") (20). Few conidia.
Trichophyton (Achorion) Schoenleinii
 3. Colonies gray or yellow. No spores. Anaerobic.
Actinomyces bovis
 4. Colonies tannish-yellow to orange; heaped up irregular folds. Aerobic.
Nocardia (Actinomyces) asteroides
 5. Colonies chalky white; short aerial hyphae.
Nocardia gypsoides
 6. Colonies light or dark and waxlike. Pear-shaped spores arranged in groups at tips of conidiophores or growing laterally along fine, branching hyphae (21).
Sporotrichum schencki
- (2) *Rapid-growing species*. Growth moist and pasty, resembling staphylococcus colonies. Yeastlike cells (budding).
- (a) Hyphae produced on corn meal agar. Ascospores (22).
Endomyces
 - (b) Hyphae produced on corn meal agar. No ascospores. Chlamydospores and cells in grapelike clusters (corn meal agar) (23). Acid and gas fermentation of dextrose and maltose; acid but not gas formation in sucrose.
Candida (Monilia) albicans
 - (c) Hyphae not produced on corn meal agar. Ascospores.
Saccharomyces
 - (d) Hyphae not produced on corn meal agar. No ascospores. Cells surrounded by large capsules (wet india-ink preparation) (24).
Cryptococcus neoformans

402. Glossary

Morphological description of the fungi necessitates the use of botanical terms unfamiliar to the bacteriologist. Some of the commoner ones are given below.

Thallus. The entire vegetative portion of the fungus.

Hyphae. The filaments composing the thallus.

Mycelium. The mass of hyphae.

Sporophores or *conidiophores*. Fertile hyphae that give rise to spores.

Spores:

Vegetative (asexual) spores:

Arthrospores and *oidia*. Formed by simple segmentation of hyphae.

(Coccidioides, Geotrichum):

Blastospores. Simple buds from cells of the thallus (*Candida*).

Conidia. Usually developed on specialized hyphae called conidiophores (*Penicillium, Aspergillus*).

Microconidia (aleuriospores). Differing from blastospores and conidia in that they are not set free when mature but remain attached until the mycelium disintegrates (*Microsporum, Trichophyton*).

Chlamydospores. Formed by condensation of the protoplasm of a cell in the thallus into a swollen body with thickened membrane; resting spores analogous to bacterial spores. May be terminal, intercalary (formed in the hypha), or lateral. (Produced by nearly all fungi.)

Macroconidia (fuseaux or spindle spores); special type of terminal chlamydospore, usually fusiform and divided into 2, 4, or 8 chambers ("ringworm" fungi).

Sexual spores:

Ascospores. An even number of spores formed in a sac (ascus) that is formed by the fusion of two parent cells of the thallus, the ascospores then being formed by mitotic division (*Saccharomyces, Endomyces, Penicillium, Aspergillus*):

Basidiospores. Similar to ascospores but, instead of being in sacs, borne on stalks called basidia (mushrooms, toadstools, and other *Basidiomycetes*).

Zygospores. Produced by fusion of two similar cells (isogamy), resulting in a thick-walled nodule.
Mucor, Rhizopus

Oospores. Similar to zygospores except that they result from the fusion of dissimilar cells (heterogamy) called the oogonium (female) and the antheridium (male).

CHAPTER 9

BACTERIAL FOOD POISONING

403. General

Bacterial food poisonings are provided by the ingestion of foods contaminated with certain types of bacteria or containing the toxic products of those bacteria. A variety of bacterial species have been suspected in outbreaks of food poisoning, of which three main types have been definitely incriminated and must be considered in analyzing an outbreak.

a. **BOTULINUM GROUP (CLOSTRIDIUM PARABOTULINUM AND CL. BOTULINUM).** These are saprophytic, gram-positive, anaerobic bacilli of the soil that produce botulism (food poisoning) only when their powerful preformed exotoxin is ingested in foods. The foods most commonly incriminated are canned vegetables, such as corn, spinach, string beans, etc. (See par. 374.)

b. **SALMONELLA (PARATYPHOID) GROUP.** Species frequently encountered are *S. typhimurium*, *S. schottmuelleri*, *S. newport*, *S. enteritidis*, and *S. choleraesuis*, which cause food poisoning either by their presence in contaminated foods in massive numbers or by rapid multiplication in the body after ingestion. The food involved may be either an improperly cooked meat from a diseased animal, sea food, or uncooked or left-over food, such as salads, custards, meat hash, prepared meats, and dairy and poultry products that have been contaminated from human or animal sources.

c. **STAPHYLOCOCCAL GROUP.** Some species of staphylococci, chiefly hemolytic strains of *Staph. aureus*, produce a powerful enterotoxin. Such foods as custards, cream fillers, and uncooked or improperly refrigerated tenderized ham are excellent media for the production of this toxin which when ingested gives rise to an acute, almost immediate, gastric disturbance.

404. Investigation Program

It is essential that the clinician, the epidemiologist, and the bacteriologist work in close cooperation on the problem of determining the cause and source of an outbreak of food poisoning. The clinician recognizes when and where food poisoning is occurring, and reports the cases and their features. The bacteriologist aids in the diagnosis, and assists the epidemiologist in determining the source of the infection by cultures of suspected

food, feces, and vomitus, food-handler examinations (if indicated), etc. The epidemiologist assembles the data, traces the source and agency, and determines corrective measures. The following general procedures are to be followed by the clinician, epidemiologist, and bacteriologist in the study of a food-poisoning outbreak:

- a. Prepare a list of all cases and their clinical features.
- b. Obtain a history of all cases, especially itemizing foods, eating places, and associations prior to the onset of symptoms.
- c. Determine the food responsible for or suspected of causing the illness by comparing histories of the cases.
- d. Study the history of the implicated food. Secure a list of the food handlers. Determine the methods of preparation and of storage of the suspected foods or food.
- e. Estimate the source of contamination by studying the history of the food.
- f. Obtain the following samples for laboratory examination:
 - (1) Left-over portions of the suspected foods (they should be packed in ice and examined promptly).
 - (2) Specimens of vomitus and feces from the cases.
 - (3) Feces from suspected food handlers (for *Salmonella* studies only).
 - (4) Specimens of blood, spleen, liver, and intestines of fatal cases.

405. Bacteriological Program

a. FIRST DAY.

- (1) Determine the morphological features of the predominating organisms in gram-stained films prepared from liquid portions of the foodstuffs or from suspensions of solid foods.
- (2) Inoculate culture media with specimens of food, feces, vomitus, etc., and incubate at 35° C. The following media should be used:
 - (a) Streak eosin-methylene blue-agar and Shigella-Salmonella agar or desoxycholate-citrate-agar plates, and inoculate a tube of selenite-F broth for the detection and isolation of organisms of the genus *Salmonella*.
 - (b) Streak blood-agar and infusion-agar plates and inoculate tubes of infusion broth for the isolation of staphylococci, streptococci, and similar organisms.
 - (c) Inoculate two tubes of fluid thioglycollate medium with samples of suspected food for growth of *Cl. para-*

botulinum or *Cl. botulinum* (if suspected, otherwise omit). Heat one of the tubes at 70° C. for 20 minutes. (The detection of these organisms in food specimens is of secondary importance. It is the presence of the toxin and not of the bacterium that is conclusive evidence that a given food is responsible for an outbreak of botulism.)

- (3) Samples of suspected foods, vomitus, gastric washings, etc., their filtrates, or filtrates of cultures may be tested for the presence of preformed toxins by oral, intravenous, or intraperitoneal administration into susceptible animals. Botulinum toxin can be demonstrated using mice or guinea pigs either by feeding or by intraperitoneal injection. Staphylococcal enterotoxin is best detected by oral ingestion of filtrates by human volunteers. Monkeys are susceptible to the toxin when administered by either the oral or intravenous route, and kittens have been used extensively for its demonstration by oral, intraperitoneal, or intravenous administration.

b. SECOND DAY.

- (1) Examine the blood-agar and infusion-agar plates for staphylococci.
- (2) Examine the plates of differential media for *Salmonella*. If present, select isolated colonies and transfer to Russell's double-sugar or Kligler's iron agar tubes for identification.
- (3) Note the presence of other organisms on all media.
- (4) Examine the anaerobic tubes. If growth is noted, prepare gram-stained films. If organisms with the morphology of *Cl. parabotulinum* or *Cl. botulinum* are found, subculture to fresh blood-agar plates and incubate these at 35° C. anaerobically for 24 to 48 hours.

c. THIRD AND FOLLOWING DAYS. Carry out determinative work on isolated micro-organisms.

CHAPTER 10

WATER AND DAIRY BACTERIOLOGY

Section I. BACTERIOLOGIC EXAMINATION OF WATER

406. Standards for Drinking Water

a. Water supplies for drinking purposes should conform to the standards prescribed from time to time by the Department of the Army. The bacteriologic tests to be run by Army laboratories are those described in the latest edition of *Standard Methods of Water Analysis*, published by the American Public Health Association. The technic herein is based on the ninth edition, published in 1946. If any major changes in procedure are incorporated in later editions, the new technic will be followed by Army laboratories.

b. Impure water may serve as a medium for the transmission of pathogenic organisms causing such diseases as typhoid and paratyphoid fevers, dysentery, diarrhea, and cholera. Water from shallow wells and from streams or lakes is especially likely to carry such organisms. All water supplied should be viewed with suspicion and not used for drinking purposes until declared potable by the proper medical officer.

407. Collection of Sample

a. Bacteriologic examination of specimens of drinking water from all Army stations is made routinely once every month and at more frequent intervals when indicated by local conditions or when prescribed. The bacteriologic examination may be supported by sanitary surveys and at times by a sanitary chemical examination of the water. These examinations are made locally when laboratory facilities are available, otherwise at Army area or other laboratory.

b.

- (1) Representative samples are collected by qualified personnel and shipped in sterile 120 ml bottles in double mailing containers to the laboratory. The collection must be carefully made to avoid extrinsic contaminating factors, such as would be added by the use of unsterile containers, tap drippings, dead-end water, insects, and other unrepresentative items. All samples of chlorinated water for bacteriologic examination must

be collected in sterile bottles to which 0.02 to 0.05 gm of sodium thiosulfate have been added. Ample air space should be left between the stopper and the level of the water sample in the bottle in order to facilitate mixing of the sample by shaking, preparatory to examination.

- (2) Since most samples of chlorinated water contain a sufficient amount of chlorine to kill the bacteria in the sample between collection and testing, it is required that such samples be collected into sterile water-sample bottles containing 0.02 to 0.05 gm of sodium thiosulfate, which rapidly eliminates the bactericidal effect of the chlorine.
- (3) The time for collecting water samples should be determined by each officer concerned after a careful examination of mail schedules and other methods of transportation in order to insure delivery in the shortest time possible. Wherever practicable, the specimen must be delivered to the laboratory on the day of collection, and preferably, samples should be taken during the first 4 days of the week.

c. Samples must be identified with the essential information concerning exact source, time of collection, special circumstances (if any), and the address of the person to whom the report is to be submitted. Indicate whether sample is *raw* or *treated* water. The laboratory includes these data in its report and adds the time of beginning examination, and the results of the test.

d. Specimens, on receipt in the laboratory, must be stored in a refrigerator and examined as soon as possible; raw (untreated) waters should be examined within 6 hours of collection, and treated waters within 12 hours.

e. Before beginning the tests, the appearance of the water (clear or cloudy) should be recorded, as well as the presence and relative amount or absence of sediment.

408. Required Tests

a. Two separate and distinct tests are run on each water sample to determine its potability: first, a total bacterial count and, second, a completed test for the presence of members of coli-aerogenes group.

b. The apparatus, materials, and media requirements are laid down in detail in *Standard Methods*, being in general those for routine bacteriologic work with special emphasis on specific details of content of media used. The media for water analysis are not

interchangeable with other bacteriologic media, as they differ in several features:

- (1) Beef extract, not beef infusion, is always used.
- (2) No sodium chloride is added.
- (3) Peptone is contained in reduced amount (0.5 percent).
- (4) The reaction is adjusted to the acid side (pH 6.4 to 7.0).

409. Total Bacterial Count

a. GENERAL.

- (1) This consists of the determination of the colony count given by 1 ml of water on standard nutrient agar or tryptone glucose extract agar after 24 hours' incubation at 37° C. It is not a true total count for it misses dead bacteria, bacteria that do not grow at 37° C., and bacteria that do not form visible colonies within 24 hours under standard conditions.
- (2) When water samples for bacteriologic examination are en route to Army laboratories in excess of 6 or 12 hours, respectively, as indicated above, the total bacterial count need not be made.
- (3) Only two portions (1 ml and 0.1 ml) of the sample are routinely plated. If an exact count of badly contaminated water is desired, additional plates may be plated with smaller measured amounts of water.
- (4) When the tests are done promptly, colony counts of over 200 per milliliter for treated waters and of over 500 per milliliter for raw waters (spring, well, etc.) are arbitrarily considered as evidencing sufficient contamination to render the water of doubtful value for drinking uses. Interpretation of any result must also consider the water source, treatment, and sanitary survey.

b. PREPARATION OF PLATE CULTURES.

- (1) Label two Petri plates with sample number and amount.
- (2) Mix the sample thoroughly by shaking vigorously 25 times.
- (3) Measure 1 ml of the sample with a sterile pipette into one plate, and 0.1 ml into the other plate.
- (4) Add to each plate 10 ml of nutrient agar or tryptone glucose extract agar that has been liquefied and cooled to 42° to 45° C.
- (5) Effect mixture of the agar and water by tilting and rotating the plates.

- (6) Allow the agar to solidify; then invert the dishes, and place them in an incubator.
- (7) Prepare a control plate by the same procedure, less the water sample.
- (8) Incubate all plates at 37° C. for 24 hours ($\pm$ 1 hour).

c. COLONY COUNTING.

- (1) Count the number of colonies on the plates, using a lens ($2\frac{1}{2}$ x) and a standard ruled counting plate or using a Quebec colony counter.
- (2) Calculate colonies per milliliter by multiplying the number of colonies in the plate by the fraction of milliliter of sample used in the plate that gives the most practical number of colonies for counting, that is, less than 300 colonies per plate. If the 0.1 ml plate gives more than 300 colonies, estimate the number of colonies present by counting at least 200 colonies covering one or more squares (one large square, or one square centimeter, on counting plate with Wolfhuegel ruling represents approximately 1/65 of the plate), and then calculating the number of colonies per plate. A 0.1 ml plate with over 10 colonies per large square need not be counted further—report “over 5,000 colonies per milliliter.”
- (3) Report colony count—exactly in low counts and approximately in higher counts—as follows:

<i>Colony count</i>	<i>Reported</i>
1 to 50	Exactly as counted
51 to 100	To nearest 5
101 to 250	To nearest 10
251 to 500	To nearest 25
501 to 1,000	To nearest 50
1,001 to 10,000	To nearest 100

410. "Completed Test" for Presence of Members of Coli-Aerogenes Group

a. GENERAL.

- (1) The “completed test” is indicated when dealing with drinking-water examination and is the usual program followed in Army laboratories.
- (2) “Partial tests” are used for hasty examinations, for raw water in process of purification, for sewage, and for other known polluted waters where completed test is unnecessary, such as in water-purification plants and sewage-disposal plants.
- (3) The coli-aerogenes group includes all aerobic and facul-

tative anaerobic, gram-negative, nonspore-forming bacilli that ferment lactose with gas formation.

- (4) Micro-organisms of this group are essentially non-pathogenic and not necessarily harmful in the water, but are considered as evidencing fecal pollution and the potential presence of pathogenic fecal organisms of the dysentery-typhoid-paratyphoid or cholera groups, which are not so readily detected in routine tests.
- (5) Positive completed tests require the demonstration, in subcultures made from initially inoculated lactose broth cultures, of one or more aerobic plate colonies of gram-negative, nonspore-forming bacilli that form gas when again inoculated into a lactose broth fermentation tube (secondary lactose tube).

b. MEDIA. The following media are required.

- (1) Lactose broth in large (30 ml) test tubes and small (10 ml) test tubes, each with small, inverted test tubes within to demonstrate gas formation.
- (2) Eosin methylene blue (EMB) agar for water.
- (3) Brilliant green lactose bile (BGLB) fermentation tubes.
- (4) Nutrient agar slant.

c. TECHNIC.

(1) *First day.*

- (a) Mix water sample thoroughly by shaking vigorously 25 times.
- (b) Inoculate two small lactose tubes with 0.1 ml and 1.0 ml portions, respectively, and five large lactose tubes each with 10 ml portions of the water sample. Label tubes with sample number, and number from 1 to 7.
- (c) Place in incubator at 37° C. for 24 hours.

(2) *Second day.*

- (a) Observe the lactose fermentation tubes. Record presence and amount, or the absence, of gas formation in column A, DA AGO Form 8-126 (Bacteriological Examination of Water).
- (b) If gas has been formed in any tubes, inoculate the following media:
 1. *EMB plate.* Plant from the gas-containing tube inoculated with the smallest amount of original water sample. The plate must be streaked to insure the presence of discrete colonies.

2. *BGLB tubes*. Plant from at least three (preferably all) tubes showing gas formation, including all such tubes inoculated with the smallest portions of original water sample.
 - (c) Place the original lactose tubes and the transplants in the incubator at 37° C. for another 24 hours.
- (3) *Third day*.
- (a) Make and record (in column B, DA AGO Form 8-126) 48-hour readings on the original lactose tubes. If no gas has been formed in any tube, report the test negative. If gas has been formed in tubes originally inoculated with smaller portions of the water sample than those showing gas at 24 hours, inoculate an EMB plate and BGLB tubes from the latter and proceed as indicated above for the second day.
 - (b) Observe the 24-hour EMB plate for typical *coli* or *aerogenes* type colonies. If present, select one or more (one of each type present) well-isolated colonies and transfer them to small lactose fermentation tubes and plain agar slants. The presence of typical colonies within 48 hours is recorded in column D on Form 8-126, as positive. If no typical colonies are found on the plate and gas has been formed in the corresponding BGLB tube, streak a new EMB plate from that tube (if no typical colonies are present on this second plate after 24 hours' incubation, fish two or more colonies considered most likely to be organisms of the *coli-aerogenes* group, transferring each to an agar slant and a small lactose broth fermentation tube). Reincubate negative plates.
 - (c) Observe the BGLB tubes for gas formation. Gas in any amount is recorded in column C on Form 8-126, as positive. Reincubate negative tubes.
 - (d) Place newly inoculated media and other media as indicated into the incubator at 37° C. for 24 hours.
- (4) *Fourth day*.
- (a) Observe all 48-hour cultures—EMB plates for typical colonies and BGLB tubes for gas formation—and record results. If positive, proceed as indicated above for the third day. If no typical colonies are present on the plates and no gas has formed in any tube of the liquid confirmatory medium, report the test as negative.

- (b) Observe the secondary lactose tubes for gas formation, and record the results in column E on Form 8-126. Reincubate, if negative.
- (c) If gas has been formed in a lactose tube, make a gram-stained film from corresponding plain agar slant. Examine for gram-negative, nonspore-forming bacilli, and record in column G on Form 8-126, as "Coliform."
- (d) If the secondary lactose broth fermentation tubes do not show gas at the end of 24 hours, examine gram-stained films from all the slants. Reincubate the lactose tubes.
- (e) When spore-forming lactose-fermenting organisms are found, the culture should be further studied to ascertain the possible presence of bacteria of the coliform group associated with the spore-bearing organisms. This may be done by transferring the culture to formate ricinoleate broth and incubating at 35° C. to 37° C. for 48 hours. If no gas is produced, only spore-forming lactose fermenters may be considered to be present. If gas is produced in the formate ricinoleate broth, the probable presence of coliform group organisms should be verified by inoculation from the formate ricinoleate to a tube of standard lactose broth and to an agar slant.

If, after 48 hours, gas is produced in the former and no spores in the latter, the test may be considered "completed" and the presence of coliform organisms demonstrated. If spores are present, for practical purposes, organisms of the coliform group may be considered absent.

(5) *Fifth day.*

- (a) Make a 48-hour reading of the secondary lactose tubes and record in column F on Form 8-126. The formation of gas in lactose broth and the demonstration of gram-negative nonspore-forming bacilli in the agar slant culture constitute a positive completed test, demonstrating the presence of a member of the coli-aerogenes group. The absence of gas formation in lactose broth or the failure to demonstrate gram-negative nonspore-forming bacilli in a gas-forming culture constitutes a negative test.
- (b) Prepare report on Form 8-126, using a standard remark (see below) wherever applicable.

In most samples of polluted water, gas formation occurs within 24 hours and the test can be completed, as shown in above outline, within 3 days of the receipt of specimen. Sometimes, as indicated in the procedures for the third day and the fourth day, lactose fermentation is delayed. In this case proceed as if fermentation had occurred within 24 hours.

411. Remarks for Reporting Results of Test

a. One or more of the following remarks should be included in the report:

- (1) Coliform bacteria not found.
- (2) Coliform bacteria in (state how many) 10-ml portions, but not 1.0 ml or 0.1 ml.
- (3) Coliform bacteria in (state how many) 10-ml portions and in 1.0 ml, but not in 0.1 ml.
- (4) Coliform bacteria in (state how many) 10-ml portions and in 1.0 ml and 0.1 ml.
- (5) Coliform bacteria in (state number) 10-ml and in 0.1 ml, but not in 1.0 ml.
- (6) *For treated waters:*
 - (a) Colony count under 200 per ml.
 - (b) Colony count over 200 per ml.
 - (c) Colony count not done—delayed in transmission.
- (7) *For raw waters.* Same as preceding, but level is 500 per ml.

b. For the interpretation of results, see current directives.

412. Differentiation of Members of Coli-Aerogenes Group

A satisfactory identification of organisms belonging to the coli-aerogenes group (for example *Escherichia coli*, *E. freundii*, and *Aerobacter aerogenes*) can be based on four tests (indol, methyl red (MR), Voges-Proskauer (VP), and sodium citrate utilization). These tests are not routinely run but are sometimes requested as part of a sanitary survey of a watershed. *E. coli* (indol +, MR +, VP —, and citrate —) is considered to be of fecal origin. *E. freundii* (indol ±, MR +, VP —, and citrate +) and *A. aerogenes* (indol —, MR —, VP + and citrate +) are considered to be less commonly of fecal origin.

413. Bacteriologic Examination of Swimming-pool Water

This is carried out using the same technic as described for drinking water. (See par. 407.)

Note. For methods of chemical analysis of water see *Standard Methods for the Examination of Water and Sewage*, latest edition.

Section II. BACTERIOLOGIC EXAMINATION OF DAIRY PRODUCTS

414. Bacteriologic Examination of Milk

a. REFERENCE. The subsequent recommendations are based on *Standard Methods for the Examination of Dairy Products* (ninth edition, 1948), published by American Public Health Association.

b. DEFINITIONS.

- (1) "Raw milk" is untreated (except for refrigeration) milk.
- (2) "Pasteurized milk" is milk that has been treated with limited heat by one of several methods, in order to kill pathogenic bacteria.
- (3) "Certified milk" is an especially pure raw or pasteurized milk, generally used for infant feeding, produced under the supervision of a medical milk commission of the county or State medical society, based on requirements of the American Association of Medical Milk Commissions.

c. GRADES OF MILK. Milk is classified (graded) in accordance with its bacterial content and with the sanitary standards under which it is produced and distributed. For grades used in the Army, see current Quartermaster specifications.

d. COLLECTION OF SAMPLES.

- (1) The sample selected should be representative of the lot to be tested, free of extrinsic contamination and so preserved by the use of sterile containers, and iced from the time of collection to the time of the laboratory test, to prevent bacterial growth in transit.
- (2) If the supply is a bottled milk, an unopened bottle may serve as a sample. The bottle should be picked at random from a distribution channel, kept on ice, and set up in the laboratory within 4 hours, if possible. The cap and lip of bottle should be protected from contamination in transmission by a tight-fitting, waterproof covering. The bottle should be packed in ice in the upright position, its temperature being kept under 45° F. until examination. If sample is to be sent to a distant laboratory by mail for a direct microscopic count, 2 drops of formalin for each 10 ml of milk should be added; a sterile 120-ml glass-stoppered bottle is filled to the stopper, labeled "Formalinized," and prepared for shipment in a double-mailing case.

- (3) Bulk milk may be sampled at the plant or during distribution by collection with sterile equipment; the sample is as above.
- (4) A plate count at a local laboratory is preferable, when properly performed, to a direct count at a distant laboratory.
- (5) All samples should be properly identified with the essential information: name of dairy, time of collection, source and grade of milk, preservative used (if any), and the address of the person to whom the report is to be submitted. The laboratory includes these data on its report and adds the time of the start of examination and the results of the test.

e. STANDARD TESTS.

- (1) *Agar plate method.* This consists of counting the number of colonies of bacteria in a culture made of a measured amount of milk or other dairy product in standard nutrient agar after 48 hours' incubation at 35° or 32° C. The count so obtained is reported as "Standard Plate Count." This count gives an estimate only of the viable bacteria present, because of clumping of bacteria in the sample, variation in growth requirements of bacteria, and other factors. The incubation temperature used in Army laboratories should be 35° C., except in examining milks purchased in States, counties, or other localities requiring incubation at 32° C. This method should be applied when an iced sample can be delivered to a nearby laboratory for immediate examination.
- (2) *Direct microscopic method.* This consists of a direct microscopic count of organisms in stained films of milk and cream. It is used in central Army laboratories in making estimates of the number of bacteria (living or dead) in specimens of formalinized milk from outlying stations where laboratory facilities for the agar plate method are not locally available. The technic is given in *Standard Methods for the Examination of Dairy Products*.
- (3) *Sediment test.* This test is performed by passing 1 pint of milk through a standard filter disk and observing for dirt, hair, and other extrinsic material. It is used as an index of the cleanness of milk.
- (4) *Tests for specific types or groups of bacteria.* Such tests, including those for the coli-aerogenes group, hemolytic

streptococci, tubercle bacilli, and *Brucella*, as described in *Standard Methods for the Examination of Dairy Products*. The tests are similar to routine laboratory examinations for these micro-organisms and, except for the coli-aerogenes group are seldom required.

f. STANDARD PLATE COUNT.

(1) *First day.*

- (a) Agitate the milk sample thoroughly.
- (b) Using sterile dilution bottles, containing exactly 9.0 ml or 99 ml of distilled water, prepare a series of dilutions depending on the expected colony count (based on grade of milk and results of previous examination). The plates to be counted should have between 30 and 300 colonies; routinely, prepare dilutions of 1:100, 1:1000 and 1:10,000.
- (c) Mix each dilution as prepared, by shaking rapidly up and down 25 times in arc of 1 foot; time interval not exceeding 7 seconds. Transfer 1.0 ml of each dilution to a properly labeled Petri dish.
- (d) Add contents of a tube of standard nutrient agar (tryptone glucose extract milk agar), previously melted and cooled to 42° C. to each Petri dish and mix with sample by carefully rotating or tilting the dish. Allow the mixture to solidify.
- (e) Incubate at 35° C. or 32° C. for 48 hours (plus or minus 3 hours).

(2) *Third day.*

- (a) Select the plates containing between 30 to 300 colonies, and count all colonies, including those of pinpoint size. The use of a Quebec colony counter is recommended. When the number of colonies in the plates exceeds 300, count representative areas of the plate and then estimate the total number of colonies present by multiplying the count secured by the proper factor. If there are between 5 and 10 colonies per large square (square centimeter) count 14 squares, selecting preferably 7 consecutive squares vertically and 7 consecutive squares horizontally across the plate. If there are more than 10 colonies per large square, count 4 of the squares, selecting preferably the 4 at the center, providing these are representative of the colony distribution. Determine the total plate count by multiplying the average number of colonies per square centimeter

by the factor, which depends on the average inside diameter of the Petri dishes being used (90 mm, multiplying by 63.5; 91 mm, by 65.0; and 92 mm, by 66.5).

- (b) Multiply the number of colonies found by the dilution factor to find the colony count per milliliters of sample.
- (c) Report: "Standard plate count _____ per milliliter at 35° C. (or 32° C)."

g. TESTS FOR PRESENCE OF "COLIFORM" ORGANISMS (COLI-AEROGENES GROUP).

- (1) *Liquid media.* Inoculate a series of fermentation tubes of brilliant green lactose bile broth with each amount of milk to be tested (10 ml, 1.0 ml, 0.1 ml, 0.01 ml, etc.). Incubate. If gas is formed, continue as with water for identification. (See par. 410.)
- (2) *Solid media.* For estimating the number of coliform organisms when present in numbers between 4 and 150 per ml, 1.0 ml of milk may be plated out in a single plate of desoxycholate agar. Follow the details of technic as described in *Standard Methods for the Examination of Dairy Products*.

415. Bacteriologic Examination of Cream

Estimations of the bacterial content of cream samples are made by using the same methods as those for milk with the following exceptions:

- a. **MEASURING SAMPLE.** Mix sample; weigh 1.0 gm aseptically into a sterile butter boat or directly into a dilution bottle.
- b. **DILUTIONS USED.** The allowable bacterial content of cream, other than certified, is greater (50,000 to 100,000 colonies per milliliter) than that for milk. Hence, the dilutions should be carried one or two steps farther when using the agar plate method.

416. Bacteriologic Examination of Frozen Desserts and Their Ingredients

a. GENERAL.

- (1) In the bacteriologic examination of ice cream, ice cream mix before it is frozen, and other frozen desserts, follow the same general methods as for milk.
- (2) Collect samples in at least 50-ml amounts in unopened cartons or in sterile, wide-mouthed, 125-ml bottles fitted with ground-glass stoppers or metal caps. In sampling

bulk ice cream, remove the top 2 to 3 centimeters with a sterile spoon, discard this, and use a second sterile spoon to collect the sample. Collect representative samples of ice cream mix at periodic intervals. Send to the laboratory immediately for examination. If the laboratory is at some distance, keep the sample properly refrigerated by packing in dry ice or in water containing cracked ice.

b. STANDARD PLATE COUNT.

(1) *Preparation of sample.* Melt the frozen dessert by placing the container in a water bath at 42° to 45° C. for a period not to exceed 15 minutes (just long enough for the contents to melt).

(2) *Methods of making dilutions.*

(a) *Volumetric.* To reduce the percentage of error when using materials of high viscosity (melted ice cream, ice cream mix, condensed milk, etc.), it is necessary to use large amounts in making the first dilution. To make a 1:10 dilution, use 11 ml of sample to 99 ml of sterile water in a dilution bottle. This method is generally used in dairy control work.

(b) *Gravimetric.* This method is more accurate and should be used for most Army laboratory examinations.

1. Immediately before measuring out the desired quantity, shake the sample container not less than 25 times to insure uniform distribution of the contents.

2. Use a sterile pipette and weigh aseptically into a sterile butter boat a 1 gm (or larger) representative portion of the frozen dessert or mix; or weigh 11 gms of the material directly into a dilution bottle, which may or may not contain 99 ml of sterile water when the sample is measured into it. The use of an 11 gm (or 11 ml) portion in 99 ml water gives a 1:10 initial dilution.

3. The butter boat or similar piece of apparatus may be sterilized in a cotton-plugged test tube. The dilution bottles must have an opening of such size as to readily take the butter boat. Remove the cotton plug from the test tube and slide the butter boat forward until it projects about 2 centimeters beyond the end of test tube. Do not allow the boat to touch a contaminated object. Weigh the test tube and butter boat

to the second decimal place. Pipette 1 gm, or slightly more, of the sample into the butter boat and again weigh to the second decimal place. Allow the butter boat and contents to slide into the dilution bottle, and add the amount of sterile water required to make a 1:10 dilution.

- (3) *Technic.* Make further dilutions, and continue as for examination of milk.

CHAPTER 11

RICKETTSIAE AND VIRUSES

Section I. INTRODUCTION

417. Definition

Viruses and rickettsiae are minute microbiological agents which are obligate intracellular parasites and are capable of causing disease. The viruses are very small when compared with other infectious agents and range in size from a few millimicrons up to several hundred millimicrons. The largest of the viruses, the elementary bodies of vaccinia and psittacosis, are readily visible in the ordinary microscope and are almost as large as certain of the smallest bacteria. Practically all of the rickettsial agents are larger than these elementary bodies and usually are about 500 millimicrons in diameter and 2,000 millimicrons in length. They therefore are about the same size as the smaller bacteria. Viruses and rickettsiae have certain biological properties usually associated with living cells. They can multiply and in the process of so doing abide by the laws of heredity. They have not been grown in the absence of living cells. Furthermore, their recognition depends upon their capacity to produce some obvious type of disease process in the host.

418. Virus and Rickettsial Procedures

Because of the complex nature of virus technics and in order to secure uniform and accurate results throughout the Army, the following procedures should be followed:

a. Wherever possible, only antigens prepared or supplied by the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Army Medical Center, Washington 12, D. C., should be employed. If antigens are prepared locally, they should be standardized at the Army Medical Service Graduate School to assure uniform results.

b. Specimens from which it is desired to attempt to isolate a virus or a rickettsial agent should be submitted to the Army Medical Service Graduate School or to the nearest Army Area or overseas theater command laboratory where adequate facilities are available. In general, the virus laboratory at the Army Medical Service Graduate School is responsible for all isolation procedures but there is no objection to the isolation of viral and rickettsial

agents in Army Area and oversea theater command laboratories provided properly trained individuals, immunized against those infections for which vaccines exist, are available.

c. When a viral or rickettsial agent is isolated, except in those instances where its nature is immediately apparent, it should be submitted promptly to the Army Medical Service Graduate School for identification in order that it may be recognized at the earliest possible time.

d. To facilitate and expedite the diagnostic procedures it is essential that a clinical abstract be submitted along with the first specimen from each case. This abstract should include the date of onset, clinical diagnosis and all pertinent clinical and laboratory data. Progress notes including additional laboratory findings should accompany subsequent specimens.

e. Direct correspondence with the laboratory on technical matters is authorized by SR 40-305-10, in order to standardize procedures, facilitate laboratory methods, control investigation, and prevent misplaced effort.

419. Collection and Shipment of Specimens for Serological Tests

In order to make a definitive diagnosis of virus or rickettsial disease by serological means, it is necessary to demonstrate an increase in antibody titer in the blood of the patient during the course of illness and convalescence. For this reason, a minimum of two specimens of serum must be obtained from each patient suspected of having virus or rickettsial disease. One of these should be collected during the acute phase of the illness. The time for collection of specimens for virus diseases is shown in table XXXVIII and for rickettsial diseases in table XXXIX. Specimens of whole blood submitted for serological tests should not be frozen and should be collected as indicated below :

a. Withdraw approximately 12 ml of blood aseptically from the patient's vein with a sterile dry syringe and needle, place in a sterile container and allow to clot at room temperature for several hours. A bleeding venule may be employed for this purpose. Place serum, obtained after separation from the clot by slow centrifugation, in a sterile Wassermann tube which is sealed with a sterile rubber stopper held in place with adhesive tape.

b. Fix a typewritten or clearly printed label to the tube giving the name of the patient and the date on which the specimen was taken.

c. Wrap the clinical abstract around the specimen and place in a mailing tube adequately packed with cotton.

Table XXXVIII. Summary of Serological Tests for Diagnosis of Common Viral Diseases of Man
with Time at which Specimens should be Collected

Disease	Type of test and time to collect specimens							
	Complement fixation		Neutralization		Test	Miscellaneous agglutinations		
	Day of bleeding		Day of bleeding			Day of bleeding		
	Early specimen (before)	Late specimen (after)	Early specimen (before)	Late specimen (after)	Early specimen (before)	Late specimen (after)		
<i>Respiratory group</i>								
Influenza A and B	—	—	—	—	RBC Aggl. = inhibition.	2	8	
Primary atypical pneumonia	—	—	—	—	Cold hemagglutinins	7	21	
Psittacosis lymphogranuloma venereum.	10	21	—	—	—	—	—	
<i>Aseptic meningitis</i>								
Lymphocytic choriomeningitis	10	21	21	42	—	—	—	
Mumps meningitis	6	21	—	—	—	—	—	
<i>Encephalitides</i>								
St. Louis	6	21	6	21	—	—	—	
Eastern equine	6	21	6	21	—	—	—	
Western equine	6	21	6	21	—	—	—	
Japanese	6	21	6	21	—	—	—	
Russian spring-summer	6	21	6	35	—	—	—	
Venezuelan	—	—	6	21	—	—	—	
West Nile	—	—	6	21	—	—	—	

420. Collection and Shipment of Specimens for Isolation and Identification of Viral and Rickettsial Agents

a. GENERAL. The attempted isolation of these agents is a laborious and expensive procedure. Most virus and rickettsial diseases can be diagnosed much more rapidly by serological means. Isolation procedures should therefore be limited to the following conditions:

- (1) Brain tissue obtained at necropsy from cases diagnosed as encephalitis, aseptic meningitis, or rickettsial disease.
- (2) Cerebrospinal fluid collected during the early febrile period only, in cases of suspected lymphocytic choriomeningitis or meningitis associated with lymphogranuloma venereum.
- (3) Blood collected during the early febrile stages only, of suspected lymphocytic choriomeningitis.
- (4) Lung tissue obtained at necropsy from cases of influenza or atypical pneumonia.
- (5) Organs obtained at necropsy if a localized outbreak of fatal disease of unrecognized etiology occurs.
- (6) Specimens of serum for antibody studies must be submitted on all living patients from whom materials are sent for isolation of virus.

b. SPECIMEN COLLECTION. Specimens submitted for isolation procedures should be frozen immediately on collection and shipped packed in sufficient dry ice to insure their arrival at the laboratory in the frozen state. They should be collected as follows:

(1) *Blood.*

- (a) As soon as a viral infection is suspected, withdraw 12 ml of blood in a dry, sterile syringe and transfer to a sterile centrifuge tube. Centrifuge promptly after clotting, withdraw serum carefully and place it in small sterile bottles or vials with rubber closures, preferably of the sleeve type. If air shipment is used, the stoppers should be secured by adhesive tape or wire. Both serum and clot should be forwarded.
- (b) Freeze contents by immersing the glass containers in a mixture of alcohol and dry ice. Rotate while freezing; this distributes the contents over a greater surface area and prevents breakage from expansion of fluid. Wrap the containers in cotton held in place with adhesive tape and pack carefully in a half gallon or gallon size vacuum bottle. Fill the remainder of the vacuum thermos bottle with small pieces of dry ice. (The dry ice may be broken up by wrapping it in a piece of cloth and then crushing with a hammer.) Cut a

**Table XXXIX. Summary of Serological Tests for Diagnosis of Rickettsial Diseases of Man
with Time when Specimens Should Be Collected**

Disease	Type of test and time to collect specimens						
	Complement fixation		Rickettsial agglutination			Weil-Felix	
	Day of bleeding		Day of bleeding		Antigen	Day of bleeding	
	Early specimen (before)	Late specimen (after)	Early specimen (before)	Late specimen (after)		Early specimen (before)	Late specimen (after)
Murine typhus.....	10	21	6	15	OX19 OX2	6	12
Epidemic typhus.....	10	21	6	15	OX19 OX2	6	12
Spotted fever.....	10	21	—	—	OX19 OX2	6	12
Q fever.....	10	21	—	—	—	—	—
Rickettsialpox.....	10	21	—	—	—	—	—
Scrub typhus.....	—	—	—	—	OXK	6	12
Fiebre Boutonneuse.....	10	21	—	—	OX19	6	12
South African tick.....	10	21	—	—	OX19	6	12

small notch down the side of the stopper of the bottle, stopper the bottle and pack carefully in a strong corrugated cardboard box.

If vacuum bottles are not obtainable, an impervious metal mailing container, such as is used for shipping ordinary bacteriological specimens, and a cardboard box may be substituted. The vial or tube containing the frozen specimen is packed in cotton in the metal container, which is then placed in a stout cardboard box filled with small pieces of dry ice and enough sawdust or similar material to fill the spaces and provide insulation. This box should be large enough to hold at least eight to ten pounds of dry ice if the shipment time is expected to be 24 to 36 hours. The box is sealed with tape and wrapped with several layers of heavy paper.

- (c) Label the package "Specimen for Bacteriological Diagnosis — RUSH — KEEP COOL," and designate that it is fragile and must not be dropped. Send this specimen and a history of the case in duplicate by air express or special delivery air mail to the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington 12, D. C. Telegraph the School when the specimen is sent so that its handling can be expedited.
- (2) *Spinal fluid.* Place about 3.0 ml of spinal fluid in each of three sterile pyrex Wassermann tubes or preferably in clean, sterile vaccine vials. Stopper, freeze, label, and ship to the Department of Virus and Rickettsial Diseases as directed above for blood.
- (3) *Autopsy material.* Perform the necropsy as soon as possible after death and collect specimens using sterile precautions. In the case of rickettsial disease, blocks of the brain and spleen tissue are forwarded. In case of neurotropic virus disease, take several blocks of about 1 cm diameter from: the gray matter of the temporal lobe, including the hippocampus; the gray matter of the frontal, parietal (motor area), and occipital cortex; mid-brain; thalamus; pons and medulla; cerebellum; cervical spinal cord.

c. PREPARATION OF MATERIALS FOR SHIPMENT.

- (1) Blocks of tissue for virus studies are shipped frozen in dry ice or unfrozen in sterile 50 percent buffered glycerin solution; the frozen method is preferable. For shipment

as frozen material, the tissues are placed in several small wide-mouth sterile bottles (without any added fluid) and stoppered with sterile rubber stoppers held in place with adhesive tape. These bottles are frozen as described above and shipped packed in dry ice in a large vacuum bottle. If the specimen cannot reach the virus laboratory in 24 to 36 hours, the material should be shipped in glycerin. For this method, place the blocks in a sterile container with at least 100 ml of sterile buffered 50 percent glycerin (see par. 2 below) and cap with a sterile rubber stopper. Ship the specimen (either frozen or in glycerin) by air express or special delivery air mail to the Department of Virus and Rickettsial Diseases, Army Medical Graduate School, Washington 12, D. C., and send a telegram at the same time. Do not send this specimen to the Armed Forces Institute of Pathology.

(2) Sterile buffered glycerin solution is prepared as follows:

- (a) Citric acid 21 gm to 1,000 ml double distilled water.
- (b) Anhydrous Na_2HPO_4 28.4 gm to 1,000 ml double distilled water.
- (c) Take 9.2 ml of (a) above and 90.8 ml of (b) above to make 100 ml of buffer solution pH 7.4, check the pH.
- (d) Mix equal parts of (c) above and C. P. glycerin; half fill cotton-stoppered specimen bottles and sterilize at 15 pounds of steam pressure for 30 minutes. Replace cotton plug with sterile rubber stopper.
- (e) In emergencies, freshly boiled double distilled water may be substituted for the sterile buffer solution in preparing the 50 percent glycerin.

421. Specimen for Histological Examination

Send duplicate blocks from each of the above listed areas of the central nervous system fixed in 10 percent formalin or Zenker's solution with other autopsy material, to the Armed Forces Institute of Pathology, Washington 25, D. C.

Section II. RICKETTSIAE

422. General

The rickettsial diseases of man are divided into five groups on the basis of clinical characteristics, epidemiology, and serology and immunological response. This grouping along with the geo-

Table XL. Nomenclature, Distribution and Transmission of Pathogenic Rickettsiae of Man

Group	Disease	Agent	Geographic distribution	Principal source of infection	Animal reservoir	Vector of man
<i>Typhus</i>	Epidemic typhus.....	<i>Rickettsia prowazekii</i>	All continents.....	Louse feces.....	Unknown.....	Body louse.
	Murine typhus.....	<i>Rickettsia typhi</i>	All continents.....	Flea feces.....	Rat.....	Rat flea.
	Brill's disease.....	<i>Rickettsia prowazekii</i>	Eastern U.S.A., Switzerland.	Unknown.....	Unknown.....	Supposed recrudescence.
<i>Spotted Fever</i>	Rocky Mountain spotted fever.	<i>Rickettsia rickettsii</i>	North America, Brazil, Columbia.	Tick bite.....	Rodents, cattle, deer, ticks.	Tick.
	Boutonneuse fever.....	<i>Rickettsia conorii</i>	Mediterranean, Central Africa.	Tick bite.....	Dog.....	Tick.
	Rickettsialpox.....	<i>Rickettsia akari</i>	New York City.....	Mite bite.....	Mouse.....	Mite.
	South African tick bite fever.	<i>Rickettsia conorii</i>	South Africa.....	Tick bite.....	Rodent (?).....	Tick.
	North Queensland tick typhus.	(?).....	Queensland.....	Tick bite (?).....	Unknown.....	Tick (?).
<i>Scrub Typhus</i>	Scrub typhus.....	<i>Rickettsia tsutsugamushi</i>	Asiatic-Pacific region.	Mite bite.....	Rodent.....	Mite.
<i>Q Fever</i>	Q fever.....	<i>Coxiella burnetii</i>	Australia, Europe, Mediterranean, U.S.A., Panama.	Tick feces, barn dust.	Cattle, goats, bandicoot.	None (inhalation).
<i>Trench Fever</i>	Trench fever.....	<i>Rickettsia wolhynica</i>	Europe.....	Louse feces.....	Body louse.....	Louse.

graphic distribution, principal source of infection, animal reservoir and vectors to man are summarized in table XL.

423. Complement-Fixation Test for Rickettsial Diseases

a. Specific complement-fixing antibodies can be demonstrated in the sera of patients convalescent from epidemic and murine typhus, Rocky Mountain spotted fever, rickettsialpox, boutonneuse fever, South African tick bite fever, North Queensland tick typhus and Q fever. The antigens of some of these agents are closely related. For example, the agents of epidemic and murine typhus have a common soluble antigen as do those of spotted fever, boutonneuse fever and rickettsialpox. Thus, a complement-fixing antigen which contains appreciable amounts of the soluble antigen common to epidemic and murine organisms can be used to diagnose infection with either murine or epidemic typhus but cannot be employed to differentiate between these two diseases. Similarly, soluble antigen from the organism of Rocky Mountain spotted fever or rickettsialpox may be used in screening tests for this group but not to differentiate the two. Specific antigens capable of differentiating murine and epidemic typhus can be prepared by repeated washing of the rickettsial organisms. This process removes the soluble antigen and leaves a highly purified rickettsial antigen. Such products are difficult to manufacture and are quite expensive. Similar purified rickettsial antigens can be prepared to differentiate between Rocky Mountain spotted fever, rickettsialpox and boutonneuse fever.

b. Q fever antigens appear to be free of soluble substance but some antigenic variation exists in the different strains of *Coxiella burnetii* so that some strains provide much broader complement-fixing antigens than others. The Henzerling and Nine Mile strains are among the best for the preparation of diagnostic antigens.

c. A wide variation exists in the antigenic composition of the various strains of *Rickettsia tsutsugamushi*. This limits the usefulness of the complement-fixation test in scrub typhus.

424. Technic for the Complement-Fixation Test

a. TITRATION OF COMPLEMENT. The complement titration is carried out with varying quantities, i.e., 0.08 ml to 0.24 ml with increments of 0.02 ml of a 1:30 dilution of fresh guinea pig serum (lyophilized commercial complement is generally used). The hemolytic system consists of equal parts of 3 percent washed sheep erythrocytes and three minimal hemolytic doses (MHD) of anti-sheep erythrocyte hemolysin. The complement-titration, made in

the presence of two units (0.25 ml) of antigen, is performed as follows:

Tube No.	1	2	3	4	5	6	7	8	9
Complement 1:30	0.08 ml	0.10	0.12	0.14	0.16	0.18	0.20	0.22	0.24
Saline 0.85 percent	0.67 ml	0.65	0.63	0.61	0.59	0.57	0.55	0.53	0.51
Antigen (2 units)	0.25 ml	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Sensitized R.B.C.	0.50 ml	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Incubate 37° C. for 30 minutes

The first tube showing complete hemolysis is taken as the exact unit and the next higher amount is a full unit. Two full units of complement, contained in 0.50 ml of physiological saline solution, are used in the tests for epidemic and murine typhus, Rocky Mountain spotted fever and rickettsialpox. (See tables XLI and XLII.) In the test for Q fever, the unit of complement is taken as the amount of complement in the fourth completely hemolyzed tube, and twice this amount contained in 0.5 ml is used in the tests. (See tables XLI and XLIII.)

Table XLI. Variable Factors in Complement-Fixation Tests for Rickettsioses

Test for—	Complement amount	Incubation tests		Serum dilutions tested
		Temperature	Period	
Typhus fever (epidemic and murine).	2 full units	4° to 8°C.	16 to 18 hrs.	1:10 to 1:640
Rocky mountain spotted fever.	2 full units	4° to 8°C.	16 to 18 hrs.	1:10 to 1:320
Rickettsialpox	2 full units	4° to 8°C.	16 to 18 hrs.	1:10 to 1:320
Q fever	2 times the amount in the 4th completely hemolyzed tube.	37°C.	2½ hrs.	1:10 to 1:320

b. TESTS WITH PATIENT'S SERUM.

- (1) The serum to be tested is inactivated at 56° C. for 30 minutes. Twenty-five one-hundredth ml amounts of antigen, at a dilution which represents two units, are added to 0.25 ml volumes of serial two-fold dilutions of serum (see table XLI for appropriate dilution range) and then 0.5 ml of diluted complement (as indicated in table XLI) is introduced. Following incubation (as shown in table XLI), 0.5 ml of the sensitized cell suspension is added. The test materials are incubated at 37° C. for 30 minutes.

when the final readings are made. Titration endpoints are estimated on the basis of the last tube showing complete or three plus fixation, and the titer is taken as the dilution of serum originally added to that tube. Known positive and negative sera are titrated to their endpoints in each test. This titration serves as a control on the activity of the hemolytic system and permits a comparison of the data obtained from one experiment to another. In addition, a complement titration in the presence of antigen is included in each test to determine the amount of complement available after incubation.

- (2) Sample protocols for performance of these tests are given in tables XLII and XLIII. Detailed instructions may be obtained upon request from the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington 12, D. C.

Table XLII. Quantitative Complement-Fixation Test for Typhus Fever, Rickettsialpox or Rocky Mountain Spotted Fever

Tube	Patient's serum 0.25 ml	Antigen 2 units	Complement 2 full units	Sensitized sheep cells
1	1:10	0.25 ml	0.5	0.5 ml
2	1:20	0.25 ml	0.5	0.5 ml
3	1:40	0.25 ml	0.5	0.5 ml
4	1:80	0.25 ml	0.5	0.5 ml
5	1:160	0.25 ml	0.5	0.5 ml
6	1:320	0.25 ml	0.5	0.5 ml
7	1:640	0.25 ml	0.5	0.5 ml
8	Serum control 1:10	0	0.5	0.5 ml

Primary incubation in refrigerator at 4° to 8° C. for 16 to 18 hrs. followed by 15 minutes at room temperature.

Secondary incubation in water bath at 37° C. for 30 min. Read.

Complement Titration (18 hours 4° to 8° C.)*

Tube	Complement 2 full units	Antigen 2 units	Saline solution	Sensitized sheep cells
1	0.1 ml	0.25 ml	0.65 ml	0.5 ml
2	0.15 ml	0.25 ml	0.6 ml	0.5 ml
3	0.2 ml	0.25 ml	0.55 ml	0.5 ml
4	0.25 ml	0.25 ml	0.5 ml	0.5 ml
5	0.3 ml	0.25 ml	0.45 ml	0.5 ml
6	0.4 ml	0.25 ml	0.35 ml	0.5 ml
7	0.5 ml	0.25 ml	0.25 ml	0.5 ml

Primary incubation 16 to 18 hrs. at 4° to 8° C.

Secondary incubation in water bath at 37° C. 30 min. Read.

* Titrate complement in presence of each antigen.

425. Interpretation of Complement-Fixation Tests

- a. The results of all complement-fixation tests are reported in

Table XLIII. Quantitative Complement-Fixation Test for Q Fever

Tube	Patient's serum 0.25 ml	Antigen (2 units)	Complement (2 units)	Sensitized sheep cells
1	1:10	0.25 ml	0.5	0.5 ml
2	1:20	0.25 ml	0.5	0.5 ml
3	1:40	0.25 ml	0.5	0.5 ml
4	1:80	0.25 ml	0.5	0.5 ml
5	1:160	0.25 ml	0.5	0.5 ml
6	1:320	0.25 ml	0.5	0.5 ml
Anticomplementary serum control				
7	1:10	0	0.5	0.5 ml
Complement control				
Tube	Complement*	Antigen (2 units)	Saline solution	Sensitized sheep cells
1	0.1 ml	0.25 ml	0.65 ml	0.5 ml
2	0.15 ml	0.25 ml	0.6 ml	0.5 ml
3	0.2 ml	0.25 ml	0.55 ml	0.5 ml
4	0.25 ml	0.25 ml	0.5 ml	0.5 ml
5	0.3 ml	0.25 ml	0.45 ml	0.5 ml
6	0.4 ml	0.25 ml	0.35 ml	0.5 ml
7	0.5 ml	0.25 ml	0.25 ml	0.5 ml

* Diluted complement used in the test above.

terms of serum titer. Four-fold or greater rise in titer during convalescence is considered of diagnostic significance.

b. Early specimens are not always available for comparative tests with the convalescent sera. Since antibodies to the rickettsiae may persist for years following infection or for some time after vaccination with these agents, the significance of a positive finding in the convalescent serum alone must be carefully examined in the light of the patient's previous history and the clinical findings. Few non-specific positive reactions occur with a 1:10 dilution of serum in the rickettsial complement-fixation test just described. However, a presumptive diagnosis based on a positive convalescent serum should not be attempted unless the titer is well above this, i.e., at least 1:40 to 1:80.

426. Agglutination Test for Rickettsial Diseases

Rickettsial agglutination tests are satisfactory for diagnostic purposes in epidemic and murine typhus and may be employed under certain special conditions in Q fever. However, since they are more difficult to perform satisfactorily and much more waste-

ful of antigen than the complement-fixation tests, their use should be limited to the Army Medical Service Graduate School in the Zone of the Interior and to Medical General Laboratories overseas. The same highly purified rickettsial antigen prepared for complement-fixation tests is used in the agglutination test but in a more concentrated form.

427. Technic for the Agglutination Test

a. Best results are obtained using 3.0 ml conical pyrex centrifuge tubes measuring 10 mm x 65 mm. It is necessary to add inactivated normal human serum (free of specific agglutinins) in sufficient quantity to give a concentration of 1:200 (0.5 percent) in the diluted antigen suspension employed in the test. This prevents spontaneous agglutination of the rickettsiae.

b. The stock rickettsial suspension is diluted to contain approximately ten complement-fixing units of antigen in each 0.25 ml. This usually represents a 1:6 to 1.15 dilution of the concentrates presently distributed by the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington 12, D. C., and approaches the turbidity of the No. 2 or 3 tube of a McFarland nephelometer. The optimal concentration of rickettsial suspension may be determined by testing serial dilutions of positive sera with several different dilutions of antigen of the approximate desired strength. The highest dilution of antigen giving maximal clear-cut serum titers is the proper dilution to use in the diagnostic tests.

c. Serial dilutions of serum are made in physiological saline solution beginning with a 1:10 dilution of serum and distributed in 0.25 ml amounts. To each dilution of serum is added 0.25 ml of rickettsial suspension. The mixture is then thoroughly shaken and placed in a water bath at 42° C. for four hours followed by storage in the ice box at 4° C. for another 16 to 18 hours when the test is read. A test is recorded as "complete" agglutination when the clumps have settled to the bottom of the tube, leaving a clear supernate, while a "partial" agglutination is recorded where definite clumping has occurred and settled out, but the supernate remains slightly cloudy. Only complete and partial agglutinations are recorded as positive and all titers represent final dilution. Positive and negative serum controls and an antigen-saline control are included with each test.

428. Interpretation of the Agglutination Test

A four-fold or greater increase in serum titer during convalescence is of diagnostic significance. Serum titers obtained by the

agglutination tests are usually 8- to 16-fold higher than the complement-fixation values. Epidemic and murine titers of 1:20 to 1:40 and Q fever titers of 1:/5 are sometimes obtained in such agglutination tests with presumably normal sera. Therefore, when only a single convalescent serum is available, it must give a positive reaction well above these limits if a presumptive diagnosis of disease is to be considered.

429. Weil-Felix Test

The Weil-Felix test continues to be of considerable importance in the early presumptive diagnosis of a number of rickettsial diseases and is the serological method of choice in scrub typhus. While it is recognized that the reaction is nonspecific, nevertheless, its simplicity warrants its use, especially in laboratories which are not equipped to make or use the specific rickettsial antigens. The test tube titration of antibody is more accurate than the slide agglutination method. Antigen is distributed by the Army Medical Service Graduate School.

430. Technic of the Weil-Felix Test

The agglutination test is performed by thoroughly mixing 0.5 ml amounts of diluted serum (inactivated at 56° C. for one-half hour) with 0.5 ml of antigen suspension. Serum dilutions of 1:10 through 1:640 (final dilution 1:20 through 1:1280) are usually sufficient. A control tube containing 0.5 ml of antigen and 0.5 ml of saline should be included as well as control tests with positive and negative human sera. Tests and controls are incubated in the water bath at 37° C. for two hours followed by storage overnight in the ice box. Complete agglutination is indicated by clearing of the supernatant fluid and by clumping of the organisms into large white particles which settle to the bottom of the tube. Partial agglutination is indicated by incomplete clearing of the supernatant fluid and diminution in size of the bacterial clumps. When

Table XLIV. Usual Weil-Felix Agglutination Reactions Observed in Rickettsial Diseases

	OX-19	OX-2	OX-K
Epidemic typhus.....	+ + + +	+	0
Murine typhus.....	+ + + +	+	0
Scrub typhus.....	0	0	+ + + +
Rocky Mountain spotted fever.....	+ + + +	+	0
Rickettsialpox.....	0	0	0
Q fever.....	0	0	0

the tubes are shaken, granular agglutinated masses of bacteria are seen.

431. Interpretation of Results of Weil-Felix Test

a. The typical responses obtained with suspensions of *Proteux* OX-19, OX-2 and OX-K organisms and convalescent sera from cases of epidemic, murine, and scrub typhus, Rocky Mountain spotted fever, rickettsialpox and Q fever are given in table XLIV. It is seen that agglutination of the OX-19 organism occurs with sera from cases of epidemic and murine typhus and of OX-K suspensions by sera from patients with scrub typhus. Similarly, the failure to obtain agglutination of any of the three organisms with sera of cases with rickettsialpox and Q fever is shown. The Weil-Felix response in Rocky Mountain spotted fever may be of several types, a high OX-19 and a low OX-2, or elevation of both OX-19 and OX-2, or occasionally, a low OX-19 and a high OX-2.

b. The Weil-Felix agglutinins may appear as early as the fifth or sixth day after onset of fever in those diseases in which the reaction becomes positive, and they are almost always present by the 12th day. These antibodies generally reach their maximum in early convalescence and then decline rather rapidly to non-diagnostic levels in one to several months. Occasional patients with typhus or spotted fever do not develop OX agglutinins; this occurs more frequently in vaccinated persons who subsequently contract typhus. It remains to be determined whether early termination of rickettsial diseases by specific antibiotic therapy has an appreciable effect on antibody levels.

c. A rise in antibody titer, demonstrated in a series of two or more sera, is essential for the presumptive diagnosis of rickettsial disease when the Weil-Felix reaction is employed. One is not justified in interpreting the results of tests on a single serum, unless the titer is high, that is, well above 1:160. The Weil-Felix reaction is of no value in differentiating epidemic from murine typhus and frequently fails to provide even presumptive evidence for separating spotted fever from typhus fever.

d. The importance of maintaining a supply of known positive human sera for rechecking the bacterial suspension at frequent intervals should be emphasized. Aging sometimes renders such antigens hyperagglutinable; this should be suspected if a number of unexplained positive results are found.

432. Isolation of *Rickettsiae*

Serologic methods are satisfactory for the routine diagnosis of the well known rickettsial diseases of man. Attempts at isolation

Table XLV. *Isolation of Rickettsiae*

Disease	Source	Preparation	A n i m a l s					
			Animal	Routes	Quantity	Incuba- tion (days)	Signs	Organ used for passage
Typhus fever, epidemic.	Human	Whole blood or macerated clot.	Guinea pig	I.P.	3-5 ml	5-12	Fever	Brain.
	Human	Whole blood or clot.	Guinea pig	I.P.	3-5 ml	3-10	Fever, scrotal reaction.	Tunica exudate, testes.
Rocky Mountain spotted fever.	Human	Whole blood or clot.	Guinea pig	I.P.	3-5 ml	3-10	Fever, illness, scrotal reaction.	Blood and spleen.
Q fever	Human	Whole blood, clot or urine.	Guinea pig	I.P.	3-5 ml	3-10	Fever	Blood and spleen.
Rickettsialpox	Human	Whole blood (inocu- lated at bedside).	White mice	I.P.	0.5-1.0 ml	6-9	Ruffed fur, inactivity.	Spleen.
Tsutsugamushi disease.	Human	Whole blood or clot.	White mice	I.P.	0.5-1.0 ml	5-12	Rough fur, ascites.	Spleen.

of rickettsiae are often unsatisfactory and are generally hazardous even to trained laboratory personnel when special precautions are taken. Therefore, under ordinary circumstances, isolation tests should not be requested by the clinician or undertaken by the laboratory. If isolation studies are indicated, specimens should be submitted according to instructions in paragraph 420. Since under critical conditions it may become necessary for inexperienced persons to attempt isolation procedures, a tabular summary of these methods is given in table XLV. Should circumstances warrant such attempts, the original inoculated animals or early passage animals should be sent promptly to the nearest Army Area or Medical General Laboratory or the Army Medical Service Graduate School for further study.

Section III. VIRUSES

433. Classification

Owing to their small size and their parasitism for living cells, it is impossible to classify viruses by their morphological characteristics alone. They are therefore generally grouped according to the predominant type of reaction produced in the animal host.

a. **NEUROTROPIC VIRUSES.** These are characterized by central nervous system involvement in the host and include, among others, the viruses of rabies, poliomyelitis, lymphocytic choriomeningitis, Western, Eastern and Venezuelan equine encephalomyelitis, Japanese encephalitis, St. Louis encephalitis, Russian spring-summer encephalitis, encephalomyocarditis and the Coxsackie group.

b. **DERMATROPIC VIRUSES.** Virus diseases characterized by skin manifestations include smallpox, vaccinia, chickenpox, herpes febrilis, herpes zoster, molluscum contagiosum, common wart, trachoma and inclusion conjunctivitis.

c. **RESPIRATORY VIRUSES.** Predominently affecting the respiratory tract, as influenza and psittacosis.

d. **MISCELLANEOUS VIRUSES.** Viruses producing generalized diffuse infections include measles, German measles, mumps, yellow fever, dengue, sandfly (*Phlebotomus*) fever and lymphogranuloma venereum.

434. Neutralization Tests for Virus Diseases

a. **GENERAL.** While diagnostic neutralization tests are not technically difficult, their proper performance requires an adequate supply of animals; a collection of standardized preparations of seed virus and specific anti-sera; the proper collection and handling of the clinical sera to be tested; and experience in interpreting

the results. Therefore, diagnostic neutralization tests should be performed only at the Army Medical Service Graduate School and in Army Area and Medical General Laboratories with proper facilities. Details of the neutralization technic recommended by the Neurotropic Virus Commission of the Army Epidemiological Board and employed at the Army Medical Service Graduate School with the encephalitic viruses are given below.

b. SEED VIRUS. Ten or more brains removed from mice showing signs of central nervous system infection are thoroughly ground with sterile sand or alundum and 9 ml of sterile diluent consisting of 10 percent inactivated normal rabbit serum in saline is added for each gram of tissue. The solution is added slowly with continuous grinding to assure an even emulsion. After centrifugation at 2,000 rpm for ten minutes, the supernatant is drawn off and considered a 1:10 dilution of virus. Part of this material is titrated immediately and the rest is distributed into ampoules in 2.0 ml amounts. The sealed ampoules are rapidly frozen in a mixture of dry ice and 95 percent alcohol and stored in a dry ice refrigerator, for not longer than two months. Depending on the needs, one or more ampoules are quickly thawed in the 37° C. water bath and used in each test, the unused portion being discarded.

c. SERA. Blood specimens are withdrawn from the patient at the time intervals specified in table XXXVIII, according to the particular disease under investigation. The sera are removed from the clot and sent at once to the laboratory. Specimens should be sterile but need not be frozen unless prolonged storage is required.

d. PREPARATION OF VIRUS DILUTIONS AND CONTROL AND SERUM-VIRUS MIXTURES. Starting with the 1:10 dilution from the frozen ampoules, 1:50, 1:500, 1:5000, etc., up to 1:500,000,000 or 1:5,000,000,000 dilutions are prepared using 10 percent rabbit serum saline as the diluent and a separate pipette for each dilution. Two-tenths ml of the selected dilutions are added to labeled tubes containing 0.2 ml of the undiluted unknown sera being tested. Following addition of serum, the final virus concentration is 10^{-2} , 10^{-3} , 10^{-4} , etc., in the example above. Both positive and negative control antisera are included in the tests.

e. SELECTION OF CRITICAL DILUTIONS.

- (1) If the preliminary titration had indicated that the 50 percent endpoint lethal titer (LD_{50}) of the virus to be used is in the range of the 10^{-8} dilution, then the virus dilutions employed in the test with normal serum will be 10^{-6} , 10^{-7} , 10^{-8} , and 10^{-9} and the dilutions used with the unknown serum will be 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} . Similarly:

Table XLVI. Summary of Common Serological Reactions Employed in Diagnosis of the Viral Diseases of Man

Group	Disease	In vitro tests			In vivo tests			Usual test animal
		Type	Common sources of—		Type	Common sources of—		
			Antigen	Antiserum		Virus	Antiserum	
Viral epidemic encephalitides.	St. Louis. Japanese. Equine: Western. Eastern. Venezuelan. Russian Spring-Summer.	Complement fixation.	Mouse brain.	Man.	Neutralization.*	Mouse brain.	Man.	Mouse.
	Other neurotropic viruses.	Complement fixation.	Guinea pig spleen.* Guinea pig lung. Mouse brain.	Man.	Neutralization. Neutralization. Neutralization.	Mouse brain.* Guinea pig brain. Mouse brain.	Man. Man. Man.	Mouse.* Guinea pig. Mouse.
Variola-vaccinia.	Smallpox (Alastrim). Vaccinia.	Complement fixation. Complement fixation.	Man-vesicle fluid or crusts. C-A membrane.* Rabbit skin. Calf skin.	Rabbit (vaccinia). Man (variola or vaccinia).	Neutralization.	Suckling mouse tissue. Mouse brain.	Mouse Man. Man.	Suckling mouse. Mouse.

* Asterisk indicates preferable test, material, or animal.

Table XLVI — Continued

Group	Disease	In vitro tests			In vivo tests			Usual test animal
		Type	Common sources of—		Type	Common sources of—		
			Antigen	Antiserum		Virus	Antiserum	
Psittacosis-lymphogranuloma venereum.	Psittacosis.	Complement fixation.*	Yolk sac*, C-A fluid.	Man.				
	Lymphogranuloma venereum.	Complement fixation.*	Yolk sac.	Man.	Antitoxin titration.	Yolk sac.	Man.	Mouse.
Influenza.	Influenza-A.	Hemagglutination-inhibition.*	C-A fluid.	Man.	Neutralization.	Mouse lung.	Man.	Mouse.
	Influenza-B.	Complement fixation.	C-A fluid.	Man.		C-A fluid.	Mouse.	Egg.
Herpetic.	Herpes simplex.				Neutralization.	Mouse brain.	Man.	Mouse.
	Epidemic keratoconjunctivitis.				Neutralization.	C-A membrane. Mouse brain.	Man. Man.	Egg. Mouse.
Mumps.	Mumps.	Complement fixation.	Monkey parotid.* Yolk sac. Amniotic fluid.	Man.				
Miscellaneous viruses.	Yellow fever.	Complement fixation.	Mouse brain.	Man.	Neutralization.	Mouse brain.	Man.	Mouse.
	Rift Valley fever.				Neutralization.	Mouse liver or brain.	Man.	Mouse.

Miscellaneous viruses (<i>continued</i>).	Dengue (Hawaiian). Colorado tick fever. Fort Bragg fever.	Complement fixation.	Mouse brain.	Man.	Neutraliza- tion. Neutraliza- tion. Neutraliza- tion.	Mouse brain. Mouse brain. Hamster brain.	Man. Man. Man.	Mouse. Mouse. Hamster.
Miscellaneous (possibly viral).	Primary atypical pneumonia (one type). Infectious mononucleo- sis.	Cold hemagglu- tination. Heterophile hemagglu- tination.	Human red cells, type O. Sheep red cells.	Man. Man.				
Miscellaneous viral diseases.	Chicken pox. Common cold. German measles. Herpes zoster. Inclusion blennorrhoea. Infectious hepatitis. Measles. Molluscum contagiosum. Poliomyelitis. Sandfly fever. Trachoma. Verruca.			NO SATISFACTORY DIAGNOSTIC SEROLOGICAL TESTS				

* Asterisk indicates preferable test, material, or animal.

- (2) With preliminary LD₅₀ titer of virus in range 10⁻⁷,
 Control dilutions 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸
 "Serum" dilutions 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶
- (3) With preliminary LD₅₀ titer of virus in range of 10⁻⁹,
 Control dilutions 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰
 "Serum" dilutions 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸
- (4) These dilutions are selected in order to be within the range of 100 percent mortality at one end and 100 percent survival at the other in the control titration, bearing in mind the variations in titer that may be expected with virus suspensions preserved in the frozen state. When the test is to be used for diagnosis with a virus that has been found to maintain its titer well, one may dispense with the lowest dilution in the serum mixtures (i.e., the 10⁻³, or 10⁻⁴, or 10⁻⁵) and use three instead of four serum-virus dilutions.
- (5) All the serum-virus mixtures are thoroughly mixed and then placed in a water bath at 37° C. for one hour. Finally, they are removed to an ice bath until inoculated into mice.

f. INOCULATION OF MICE. Groups of six 12 to 14 gram mice of proven uniform susceptibility are inoculated intracerebrally with 0.03 ml amounts of the serum-virus mixtures using 0.25 ml tuberculin syringes equipped with 25 gauge 1/4" needles. A single syringe may be used for injecting each serum provided that the highest dilution of virus is inoculated first, followed in turn by the successively lower dilutions of virus. The syringe must be emptied after each dilution and the inside thoroughly rinsed by filling and discharging the next lower dilution.

g. OBSERVATION OF MICE. The mice must be observed daily for symptoms of central nervous system infection to establish the specificity of deaths. The mice inoculated with Western and Eastern equine encephalomyelitis should be observed for 10 days and with the remaining viruses for 21 days. All deaths within 24 hours after inoculation shall be regarded as due to traumatic or non-virus causes.

h. DETERMINATION OF NEUTRALIZATION INDEX.

- (1) First the LD₅₀ for each serum is determined by the Reed and Muench formula in the following manner:

- (a) Set up protocol and compute percent mortality for each dilution as in the example below :

Virus Dilution	No. of mice		Accumulated totals		
	Died	Survived	Died	Survived	Percent mortality (each dilution)
10 ⁻⁶	6 ↑	0 ↓	13	0	100
10 ⁻⁷	5 ↑	1 ↓	7	1	87.5
10 ⁻⁸	2	4 ↓	2	5	28.6
10 ⁻⁹	0	6 ↓	0	11	0

¹ 50 percent.

Arrows indicate direction of addition for computing accumulated totals.

- (b) Calculate in the following manner the proportionate distance between the two log dilutions (in the example cited 10⁻⁷ and 10⁻⁸) where the 50 percent endpoint will fall:

% mortality above 50% - 50%

$$\frac{\% \text{ mortality above } 50\% - 50\%}{\% \text{ mortality above } 50\% - \% \text{ mortality below } 50\%} = \frac{87.5 - 50}{87.5 - 28.6} = \frac{37.5}{58.9} = 0.64$$

Log LD₅₀ titer = Log of dilution above 50% + factor
or in this case = 7.0 + 0.64 = 7.64

$$LD_{50} = 10^{-7.64}$$

- (2) Calculation of neutralization Indices.

Log of neutralization index = Log of LD₅₀ titer of control minus the Log LD₅₀ titer of serum.

Neutralization index = antilog of difference.

Thus for example:

Serum	LD ₅₀	Log of neut. index	Neut. index (antilog)
Negative control.....	10 ^{-8.5}		
Positive control.....	10 ^{-4.5}	4.0	10,000
Acute specimen.....	10 ^{-7.5}	1.0	10
Convalescent specimen.....	10 ^{-5.5}	3.0	1,000

In the above example, the acute phase specimen neutralized 10 LD₅₀ of virus and the convalescent serum neutralized 1,000 LD₅₀, hence, there was a hundred-fold rise in neutralizing titer during convalescence.

435. Interpretation of Neutralization Tests

To be of diagnostic significance, there should be an increase in neutralizing capacity of at least 100 LD₅₀ during convalescence. Where only one convalescent serum specimen is available, a posi-

tive neutralization test is of little value except when the antibody titer is quite high. Neutralizing antibodies for the neurotropic viruses persist for long periods of time, therefore, unless a rise in titer is demonstrated in paired sera, one cannot be sure that the immune substance did not arise from an earlier infection which may have been inapparent.

436. Complement-Fixation Tests for Virus Diseases

The basic principles of the complement-fixation technic are identical whether employed in the diagnosis of virus diseases or elsewhere. It is difficult, however, to prepare suitable antigens from certain viruses and the test is subject to technical errors. To assure uniform results wherever possible, only antigens prepared or supplied by the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, should be employed. If antigens are prepared locally, they should be standardized at the Army Medical Service Graduate School before use in routine diagnostic work.

437. Complement-Fixation Test for the Psittacosis-lymphogranuloma Venereum (Psitt-LV) Group of Virus Infections

a. This test has proven very reliable in practice and stable antigens for its performance are available to all the Army Area and Medical General Laboratories.

b. The psitt-LV group is made up of a number of viruses which are morphologically and antigenically similar. These agents occur as frank or latent infections in a variety of mammalian and avian species and give rise to several clinical entities in man. The commonest of the agents of this group which infect man are the viruses of lymphogranuloma venereum, psittacosis, and ornithosis (pigeon psittacosis).

c. The complement-fixation test for infection with viruses of the psitt-LV group is similar to that for the rickettsiae except for minor modifications.

- (1) The hemolytic sytem is standardized and the complement titrated as described for the rickettsiae except that saline solution is substituted for antigen in the complement titration. Two full units of complement are employed in the tests. Patient's serum, complement and antigen are contained in 0.25, 0.50 and 0.25 ml, respectively.
- (2) The primary incubation period is at 37° C. for 1¼ hours and the final incubation is at 37° C. for ½ hour.
- (3) Each serum is tested with normal control antigen as well as the psitt-LV group antigen. The viral and control

antigens are supplied by the Army Medical Service Graduate School and are to be diluted 1:4 with physiological saline solution for use in the tests.

- (4) The detailed protocol for a sample test and its appropriate controls is shown in table XLVII.

438. Interpretation of the Complement-Fixation Test for Diagnosis of Psitt-LV Group

a. A four-fold or greater rise in antibody titer during the course of illness is of diagnostic significance. In some cases, however, an early specimen may not be available and in these instances interpretation of the results of the complement-fixation test is based on titer of the serum.

b. A serum titer of 1:40 or greater is considered to be evidence of old or recent infection with a member of the psitt-LV group of viruses.

c. A serum titer of 1:20 is of little diagnostic value since sera from presumably normal individuals occasionally show titers of this order of magnitude when tested by this method.

d. Titers of less than 1:20 are of no diagnostic significance.

e. Complement-fixing antibodies against the psitt-LV viruses generally appear in detectable amounts in the sera of infected persons within 3 to 5 weeks after onset of disease. They increase to a maximum within a few weeks after their appearance, often reaching titers of 1:320 or 1:1280. These antibodies generally persist for a number of years, being detectable in lower titers. Because of the persistence of complement-fixing antibodies in infections with this group of viruses, it is essential, in order to establish the casual relation of a member of this group to a given illness, to demonstrate a rise in antibody titer during convalescence.

f. There should be no fixation of complement with the normal control antigen. If this does occur, one should suspect the presence in the serum of syphilitic antibody or antibodies against chick embryo tissue; the serum should be submitted for a routine Kahn test. A positive reaction with control antigen is infrequent and when present, the titer is usually below 1:20. In such cases, unless the titer obtained with the psitt-LV antigen is at least four tubes (16 times) higher than that obtained with the control antigen, the test is of no diagnostic value and the serum must be reported as unsuitable for diagnosis by this test. If the titer obtained with the virus antigen is four tubes (16 times) or more greater than that obtained with the control antigen, then an interpretation can be given but it is still desirable to ask for another specimen of serum for repeating the test.

g. It must be remembered that because of the antigenic simi-

Table XLVII. Quantitative Complement-Fixation Test for Psittacosis-Lymphogranuloma Venereum Group Infection

Row	Tube No.	Patient's serum 0.25 ml	Complement 2 full units	Paitt-LV antigen 2 units (1:4)	Normal control antigen (1:4)	Saline solution	Sensitized sheep cells	Remarks
2	1	1:10	0.5 ml	0.25 ml	0	0	0.5 ml	Test proper.
	2	1:20	0.5 ml	0.25 ml	0	0	0.5 ml	
	3	1:40	0.5 ml	0.25 ml	0	0	0.5 ml	
	4	1:80	0.5 ml	0.25 ml	0	0	0.5 ml	
	5	1:160	0.5 ml	0.25 ml	0	0	0.5 ml	
3	1	1:10	0.5 ml	0	0.25 ml	0	0.5 ml	Normal antigen control.
	2	1:20	0.5 ml	0	0.25 ml	0	0.5 ml	
	3	1:40	0.5 ml	0	0.25 ml	0	0.5 ml	
4	1	1:10	0.5 ml	0	0	0.25 ml	0.5 ml	Anticomplementary controls.
	2	1:20	0.5 ml	0	0	0.25 ml	0.5 ml	
	3	1:40	0.5 ml	0	0	0.25 ml	0.5 ml	
Hemolytic system controls								
Anticomplementary antigen controls.			0	0	0	1.0 ml	0.5 ml	Should show no hemolysis (4+).
			0.5 ml	0	0	0.5 ml	0.5 ml	Should completely hemolyze (0).
			0.5 ml	0.5 ml	0	0	0.5 ml	Should completely hemolyze (0).
			0.5 ml	0	0.5 ml	0	0.5 ml	Should completely hemolyze (0).
			0.5 ml	0	0	0.5 ml	0.5 ml	Should completely hemolyze (0).

Note. Run known positive paitt-LV group infection and normal control sera as above.

larity of the strains, the complement-fixation test for infection with the psitt-LV group of viruses is entirely group specific. Thus, the sera from patients infected with any of the agents of the group will react with the antigen supplied by Army Medical Service Graduate School and the same antigen is used for the diagnosis of psittacosis and for lymphogranuloma venereum.

439. Agglutination-Inhibition Test

The following technic for the diagnosis of influenza has been agreed upon by the Commission on Influenza of the Armed Forces Epidemiological Board and the Department of Virus and Rickettsial Diseases of the Army Medical Service Graduate School.

(a) MATERIALS REQUIRED.

- (1) Saline solution (0.85 percent NaCl in freshly boiled distilled water).
- (2) Type "O" human red cells, either fresh or preserved in Alsever's solution.
- (3) Standard type A (PR8), type A prime (FM1) and type B (Lee) antigens, either in the form of merthiolated allantoic fluid or lyophilized.
- (4) Standard chicken antisera against each of the viruses mentioned in *a* (3) above.
- (5) Kahn tubes (12 x 75 mm) with evenly rounded bottoms.
- (6) Racks for Kahn tubes (flat bottom type without depressions).
- (7) One ml serological pipettes, graduated in 0.01 ml, and 5.0 ml pipettes, graduated in 0.1 ml.

b. PRECAUTIONS. Glassware must be chemically clean since traces of acid or alkali interfere with the test. Kahn tubes which do not have evenly rounded bottoms should be discarded. It is advisable to keep specially selected and cleaned tubes set aside for use only in influenza work.

c. STANDARD ERYTHROCYTE SUSPENSION.

- (1) The standard suspension consists of 0.5 percent human type "O" erythrocytes in physiological saline solution. The blood may either be freshly collected or preserved (with sterile precautions) in Alsever's solution for not longer than 1 month at 5°C.
- (2) The blood is processed as follows: The cells are washed three times in saline solution. Following the last wash, the sedimented cells are transferred to a 15.0 ml graduated centrifuge tube. The volume of packed cells is determined by centrifuging at 1,500 rpm for 10 minutes in a centrifuge equipped with a horizontal head having a radius of 5½ inches (i.e., 5½ inches from axis to trunnion slot).

- (3) The volume of cells is noted and the supernatant fluid is withdrawn and discarded. A one-half percent (0.5 percent) cell suspension is prepared by adding 199 volumes of saline solution for each volume of packed cells (e.g., 3.0 ml packed cells $\times$ 199 = 597.0 ml of saline solution to add to give a 0.5 percent suspension).

d. STANDARD ANTIGENS.

- (1) Standard influenza virus antigens, type A (PR8), type A prime (FM1) and type B (Lee), are supplied by the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School. These consist of merthiolated allantoic fluid from infected eggs and are shipped in lyophilized form. The titers of the antigens at the time of preparation are stated on the labels.
- (2) The dried antigens are to be rehydrated by adding the quantity of sterile (freshly boiled) distilled water indicated on the label. Care must be taken to incorporate all the dried material in the fluid and sterile procedures must be followed to prevent contamination. In order to obtain complete dispersion and full activity, the vial (stoppered with a small sterile cotton plug) should first be allowed to stand at 4° C. (with frequent shaking) for 2 hours after addition of the distilled water. Then the material is thoroughly dispersed with a 5.0 ml syringe and 20 gauge needle by forcefully squirting the fluid in and out of the syringe a half-dozen times. The needle point should be kept beneath the fluid in the vial in order to avoid excessive foaming.
- (3) The rehydrated material should be transferred to a sterile container (a vaccine bottle is satisfactory) and tightly stoppered. Such antigens should have hemagglutinating titers of 1:160 or higher. The antigenicity is retained better when the rehydrated stock virus is maintained at -20°C. A precipitate comprising soluble salts (phosphates and urates) and non-soluble material forms on storage at refrigerator temperature. A variable amount of virus is adsorbed to or occluded by the precipitate. The virus must be freed from the precipitate since viral activity is conditioned by the degree of dispersion and this in turn conditions the unit of the antigen used in the test. The following procedure should be followed:
 - (a) Shake the vial of antigen vigorously until a relatively homogenous suspension is effected. Remove a representative sample of desired volume, using sterile precau-

tions to prevent contamination of the vial of antigen, and dilute the amount required for the day's work with saline solution at room temperature to give a 1:20 dilution. Heating the diluted material at 37° C. for 20 minutes effects complete solution of the salts and an antigen so treated will function satisfactorily in the test.

- (b) Better restoration of activity, however, may be effected by letting the diluted antigen stand at room temperature for 1 hour to provide for maximal elution of virus from the insoluble precipitate. The latter procedure should be followed whenever possible.
- (4) A sufficient amount of the 1:20 dilution of antigen for the day's work is prepared as described in the preceding paragraph. This diluted antigen is to be titrated to determine the proper dilution for its use in the tests.
- (5) The rehydrated stock antigen will continue to have a hemagglutinating titer of 1:160 or greater for a number of weeks or months if stored at 4° C. or - 20° C. when not in use and if warming to 37° C. (as recommended for diluted stock) is avoided. Stock antigens with titers below 1:160 should not be used.

e. **TITRATION OF ANTIGENS.** The hemagglutinating unit of antigen is defined as 0.5 ml of the highest dilution of antigen which agglutinates the 0.5 ml of standard human erythrocyte suspension completely. The unit is to be determined as follows:

- (1) Set up three rows of Kahn tubes, ten tubes in each row. Place 0.5 ml of saline in tubes two through ten of each row. Add 0.5 ml of the 1:20 dilution of PR8 antigen to the first and second tubes of the first row. Mix the contents of the second tube thoroughly and transfer 0.5 ml to the third tube of the first row. Continue until all dilutions have been completed and discard the last 0.5 ml following mixing in the tenth tube.
- (2) In a similar manner prepare serial dilutions of FM1 virus in the second row of tubes and Lee virus in the third row of tubes. A single pipette can be used to make the dilutions in each row, but a different pipette must be used for each of the three rows. This procedure provides serial dilutions of each virus in 0.5 ml quantities from 1:20 through 1:10,240.
- (3) To each tube add 0.5 ml of 0.5 percent erythrocyte suspension.
- (4) Include an erythrocyte control comprising 0.5 ml of saline solution and 0.5 ml of the erythrocyte suspension.

- (5) Mix well by shaking.
- (6) Let stand at room temperature (22° to 25° C.) for 1¼ hours. Care must be taken not to bump or disturb the tubes during this incubation period.
- (7) Read and record the tests on the basis of pattern:
 - (a) Positive agglutination (+) is indicated by a red, granular, diffuse lining of the bowl of the tube.
 - (b) Absence of agglutination (O) is indicated by the formation of a compact red "button" at the bottom of the tube.
 - (c) Partial agglutination (+—) is indicated by something in between a diffuse lining of the bottom of the tube and a "button." This takes the form of a ring with a hollow center. The rings are of varying size depending upon the extent of agglutination. (Not to be confused with partial agglutination is "slippage," which is especially manifest in the test proper in the tubes containing high concentrations of serum. In this instance, the agglutinated cells do not cling to the bowl but rather slip toward the bottom and eventually form a compact mass indistinguishable from a "button." After 1¼ hours, however, "slippage," if it occurs at all, is easily recognizable since it is only partial and the edges are "ragged" or "feathery.")

f. **TEST ANTIGENS.** Each antigen for use in the test is to contain 4 units in 0.25 ml. This will be 1/8 the highest dilution of antigen which showed complete agglutination in the antigen titration. (Note that, in the test, the 4 units of antigen are contained in 0.25 ml. In the virus titration, the antigen dilutions were 0.5 ml in volume, two times as great.)

Example:

$$\text{Titer} = 1:320$$

$$\text{Test antigen (containing 4 units in 0.25 ml)} = 320 \div 8 = 1:40$$

In the example just cited, the proper dilution for use in the test would be a 1:2 dilution of the 1:20 stock dilution of the standard antigen which was described in paragraph d.

g. **THE DIAGNOSTIC AGGLUTINATION-INHIBITION TEST.** Two serum specimens from each patient must be tested simultaneously with all three types of antigen. The first of these (acute phase) is obtained within 2 to 3 days after onset of illness and the second (convalescent) is obtained 10 to 14 days after onset of illness. The tests are set up as follows:

- (1) Three-tenths (0.3) ml amounts of the sera to be tested are inactivated at 56° C. for 30 minutes. They are then diluted 1:8 by adding 2.1 ml of saline solution.

- (2) For each serum to be tested set up three rows of Kahn tubes, preferably in a rack with three rows of ten holes each. Three-fourths (0.75) ml amounts of saline solution are added to tube numbers two through ten in the front row. Number one tube (far left) in the front row and all the tubes in the middle and back rows are left empty. Twenty-five-one-hundredth (0.25) ml amounts of the inactivated 1:8 dilution of serum are transferred to tube one of each of the three rows and 0.75 ml of this same dilution of the serum are placed in tube number two of the front row. This is mixed thoroughly and 0.25 ml are transferred to the second tube of the middle and back rows and 0.75 ml are placed in the third tube of the front row. The operation is repeated in this manner until three identical series of two-fold dilutions have been prepared. Each tube contains 0.25 ml and the serum dilutions range from 1:8 to 1:4096.
- (3) After the serum dilutions have been prepared, 0.25 ml amounts of PR8 test antigen (see par. f) are added to the tubes in the first row, 0.25 ml amounts of FM1 antigen are added to the second row and 0.25 ml amounts of Lee antigen are added to the third row.
- (4) Shake well.
- (5) To the serum-antigen mixtures add 0.5 ml of the 0.5 percent erythrocyte suspension.
- (6) Shake well.
- (7) Incubate at 22° to 25° C. for 60 minutes, being careful not to bump or move the racks. [A shorter (1 hour) incubation period is used for the test than for the titration (1¼ hrs.) because serum accelerates sedimentation of cells.]
- (8) The tests are read and recorded in terms of degree of inhibition of agglutination by each serum using each antigen. The titer of a given serum using a given antigen is defined as the highest dilution of serum which effects complete inhibition of agglutination.

h. CONTROLS.

- (1) *Standard antisera.*
 - (a) Antisera from roosters immunized against the PR8, FM1 and Lee strains of influenza virus will be used for control purposes. These sera are dried in 0.5 ml volume and the contents of each vial are to be rehydrated sterilely with 5.0 ml (a 1:10 dilution of the original serum) of sterile, distilled water containing 1:10,000 crystalline merthiolate which will be supplied

with the serum. These serve as stock dilutions of standard sera and should be transferred to a sterile, tightly stoppered container after rehydration and stored at 4° C., or preferably at -20° C. These sera were inactivated prior to drying and should not be inactivated again.

- (b) Three-tenths (0.3) ml amounts of the stock dilutions of standard chicken antisera are diluted further by adding 2.7 ml of saline solution to each. The resultant mixture represents a 1:10 dilution of the stock 1:10 dilution and is a 1:100 dilution of the original rooster serum. These standard chicken sera are diluted and tested in the manner already described for the test sera, except that a 1:100 initial dilution of chicken serum is used instead of the 1:8 dilution of human serum described. The titers of these sera are stated on the original labels on the lyophilized vials of serum. The sera are highly specific and there should be no crossing between type B and type A or A prime virus. This means that type B serum should not inhibit agglutination of cells by type A or A prime virus, and conversely, type A and A prime sera should not inhibit agglutination of type B virus. Type A and A prime may cross in the first tube, that is, type A antiserum diluted 1:100 may inhibit agglutination by type A prime virus, and conversely, type A prime antiserum may inhibit agglutination by type A virus at the same dilution of serum. Inhibition of either type A or type A prime virus by heterologous antiserum at a dilution greater than 1:100 should not occur.
 - (c) The tests with positive control sera serve to confirm the identity of the antigen used and to further test the specificity and quantity of the reacting materials in the test antigens. The titer of a given serum is inversely affected by changes in the concentration of virus used in the test. The titer of the positive control serum is more regularly reproducible than that of the virus and the titrations of the standard sera are of considerable value in correlating the results of tests made on successive days. The titer of a given antiserum should not vary more than a single two-fold dilution from that obtained in previous tests.
- (2) *Retitration of test antigens.*
- (a) The test antigens must be retitrated at the same time that the agglutination-inhibition tests are performed.

This is done in the manner described for the titration of antigens (*e* above). The initial dilution will, however, be the dilution of the test antigen (1:40 in the example). The serial dilutions of this material need only be carried through seven tubes.

- (b) These retitrations of antigen are read after 1¼ hours' incubation at 22° to 25° C. Complete agglutination should occur in the first four tubes of each antigen tested and there should be less than complete agglutination in the fifth and remaining tubes.

440. Interpretation of Agglutination-Inhibition Tests

a. Results are reported in terms of the titers of each of the paired sera obtained with each antigen. A four-fold or greater increase in titer during convalescence is considered of diagnostic significance. However, if only a four-fold increase in titer is obtained with a given pair of specimens, it is best to repeat the test immediately to confirm these results before reporting.

b. The agglutination-inhibition test for the diagnosis of influenza is employed for two purposes:

- (1) To establish the presence of a given type of influenza virus in a certain area at a specific period of time.
- (2) To establish a specific diagnosis of influenza in a given individual.

c. From the point of view of preventive medicine in the Army, the first ((1) above) is of vital importance. However, it is also important to diagnose influenza infection when it occurs in individual patients during a non-epidemic period, since this may be the first indication of the beginning of a widespread outbreak. Thus, the early diagnosis of individual cases may give warning of a possible epidemic so that appropriate preventive measures can be instituted.

d. In larger outbreaks of influenza, it is usually not feasible to make an individual laboratory diagnosis on each patient. Individual diagnoses on a representative group of individuals are usually sufficient to establish the existence of an epidemic of influenza. It should also be pointed out that not all cases of influenza in a given outbreak are to be expected to show a diagnostic rise in serum titer.

e. In interpreting the results of tests with paired sera showing a diagnostic rise in antibody titer, it is important to keep in mind and investigate, if necessary, the possibility of recent (3 to 4 weeks) vaccination for influenza. Immunization may have been undertaken locally or generally throughout the Army and in some

Table XLVIII. Outline of Methods for Primary Isolation of Viruses.

Virus	Type of material to be submitted		Isolation of virus		Animal passage		Pathology
	Clinical	Autopsy	Common animals used in order of preference	Route of inoculation	Tissue	Route of inoculation	
Rabies		Brain	Mouse, guinea pig, rabbit.	I.C.	Brain	I.C.	Negri bodies.
Lymphocytic choriomeningitis.	Blood	Brain	Mouse, guinea pig	I.C.	Brain	I.C.	
Equine encephalomyelitis	Spinal fluid			I.P.	Spleen	I.P.	
	Blood	Brain	Mouse, guinea pig, rabbit.	I.C.	Brain	I.C.	
Herpes	Spinal fluid	Brain	Mouse, guinea pig, rabbit, egg.	I.C.	Brain	I.C.	Inclusion bodies, (intranuclear).
Lymphogranuloma venereum	Spinal fluid, pus from bubo.	Spinal fluid, Brain	Mouse, guinea pig	CAM	Brain	I.C.	
				I.C.			
St. Louis encephalitis		Brain	Mouse, hamster	I.C.	Brain	I.C.	
Japanese encephalitis	Blood	Brain	Mouse, hamster	I.C.	Brain	I.C.	Purkinje cell degeneration.
West Nile encephalitis	Blood	Brain, blood(?)	Mouse, hamster	I.C.	Brain	I.C.	
Russian spring-summer encephalitis.	Blood	Brain	Mouse	I.C.	Brain	I.C.	
(Also louping ill.)							
Poliomyelitis	Spinal fluid	Blood(?)		I.P.	Central nervous system.	I.P.	Ant. horn cell degeneration.
	Feces	Cord	Monkey	I.C.		I.C.	
		Medulla		I.N.			
Coxsackie group	Feces, nasal washing.		Suckling mice	I.P.	Brain, muscle	I.P.	

Yellow fever	Blood	Viscera	Monkey, mouse	I.C.	Brain	I.C.	Hepatic necrosis.
		Brain		I.P.	Viscera	I.P.	
Psittacosis	Sputum	Lung	Mouse, egg	I.P.	Viscera	I.P.	Inclusion bodies (cytoplasmic).
	Blood	Blood		Y.S.	Yolk sac		Elementary bodies.
Variola	Vesicle fluid crusts.	Vesicle fluid crusts.	Egg	CAM	Chorioallantoic membrane.	CAM	Elementary bodies, pocks.
Influenza	Nasal washings.	Lung	Egg, ferret, mouse	Am.S., I.N., Ca.S.	Embryonic fluids, nasal mucosa and lung.	Am.S., I.N., Ca.S.	Pneumonitis.
Mumps	Saliva Spinal fluid		Monkey, egg	Parotid Am.S., Y.S.	Amniotic fluid Parotid, yolk sac.	Parotid Am.S., Y.S.	Parotitis.

I.C.—Intracerebral; I.P.—Intraperitoneal; I.N.—Intranasal; CAM—Chorioallantoic membrane; Ca.S.—Chorioallantoic sac; Am.S.—Amniotic sac.

instances military personnel have received influenza vaccine at civilian institutions. A rise in titer to both type A and type B virus should be expected of being due to recent vaccination and a thorough check should be made. Concurrent infection in a single individual with both type A and type B influenza virus is a rare occurrence.

441. Cold-Agglutinin Test

The cold hemagglutination reaction is a non-specific test which can be demonstrated in approximately 55 percent of cases of primary atypical pneumonia. This is one of the simplest tests for viral disease and can be performed in nearly any laboratory. The technic for the test is given in paragraph 309.

442. Isolation of Viruses

a. General remarks on the isolation of viruses are similar to those given under paragraph 432. Major dependence is placed on serological tests but isolation procedures are used somewhat more frequently than in the rickettsial diseases. See paragraph 420 for types of material to be submitted. In sharp outbreaks of influenza-like disease, nasal washings should be obtained from two or three representative cases during the first 24 to 36 hours after onset and shipped in dry ice to the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington 12, D. C. Paired sera from these same cases should also be sent. Such studies are not primarily for diagnosis in the individual patient, since serological tests are adequate for this purpose, but are for special antigenic analysis of the epidemic strain of virus in connection with continuing work on influenza vaccine. Autopsy material from any sharp outbreaks of undiagnosed fatal disease should be submitted for study.

b. Isolation of viral agents should not be attempted by the inexperienced except as an emergency measure. If undertaken the inoculated original or early passage animals should be sent to the nearest Army Area or Medical General Laboratory or the Army Medical Service Graduate School for identification. A tabular summary of the basic information needed for isolation attempts is given in table XLVIII.

CHAPTER 12

SERODIAGNOSIS OF SYPHILIS

443. General

a. The cardiolipin microflocculation test and the cardiolipin complement-fixation test (a cardiolipin antigen standardized for use in the Kolmer technic) are outlined for the examination of sera. The cardiolipin microflocculation technic is recommended for quantitative determinations. The cardiolipin complement-fixation technic is recommended for the examination of spinal fluids.

b. The colloidal gold test with spinal fluid is described. Although the shape of the curve is of no diagnostic significance, the number of tubes showing precipitation provides a rough measure of globulin content of the fluid.

444. Cardiolipin Complement-Fixation Test with Serum and Spinal Fluid

a. GLASSWARE AND APPARATUS.

(1) *Pipettes*. The following pipettes are recommended:

1 ml, graduated in 0.01 ml (to tip)

5 ml, graduated in 0.10 ml (to tip)

10 ml, graduated in 0.10 ml (to tip)

(2) *Test tubes*. Test tubes measuring 10 by 1.3 cm (inside diameter) with rounded bottoms and no lips are employed.

(3) *Cylinders*. Graduated (100 and 250 ml) cylinders are used for measuring amounts over 50 ml.

(4) *Test-tube racks*. Metal Wassermann Test-tube Support, 20 tube.

(5) *Water bath*. Any easily regulated water bath that can be used satisfactorily at 56° or 37° C. is suitable.

(6) *Refrigerator*. Any refrigerator maintaining a temperature of 6° to 8° C. is satisfactory.

b. REAGENTS.

(1) *Kolmer saline solution*. Dissolve 8.5 grams of dry, chemically pure sodium chloride and 0.1 gram of anhydrous magnesium sulfate in 1000 ml of freshly distilled water. If the sodium chloride has absorbed moisture it should be dried in the hot air oven (160° to 200°C.) for 15 or 20 minutes and allowed to come to room temperature before weighing. Salts may be weighed and dispensed into corked tubes to avoid daily weighing. Filter the solution through filter paper into a flask and stopper

with a gauze covered cotton stopper. Fresh saline solution should be prepared the day tests are started.

- (2) *Sheep corpuscles.* Three hundred to four hundred ml of blood may be collected from the external jugular vein of a sheep into a sterile container with 60 ml of sterile 5 percent sodium citrate as an anti-coagulant. Sheep cells collected in this manner usually remain satisfactory for two weeks if properly refrigerated. A preferable method is to collect the blood under sterile conditions into a container with modified Alsever's solution, composed of 2.05 percent dextrose, 0.42 percent sodium chloride, 0.8 percent tri-sodium citrate, and 0.055 percent citric acid. A rubber stoppered bottle is filled to the 150 ml mark with the Alsever's solution, evacuated by suction, and then sterilized for 15 minutes at 15 pounds pressure in an autoclave sterilizer. The sterilized solution should have a pH of 6.1 and show little or no evidence of caramelization (indicated by a light yellow coloring of the solution). A 20 inch length of $\frac{3}{16}$ inch inside diameter, $\frac{3}{32}$ inch wall, latex tubing fitted with a screw clamp and two 15 stub gauge needles is sterilized and used for collecting the sheep's blood into the sterile evacuated bottle. After shearing an area over the jugular vein and painting with tincture of iodine, the clamp is tightened on the tubing, one needle inserted through the rubber stopper and the other needle inserted into the animal's vein. As soon as blood is observed in the tubing the clamp should be loosened to permit a rapid flow of blood into the bottle which should be rotated constantly during the operation. When the bottle is filled to the 300 ml mark the screw clamp is tightened and the needles withdrawn from vein and bottle. Blood remaining in the tubing should be discarded. Blood and preservative should be thoroughly mixed and stored immediately at 6° to 8° C. For convenience the blood may be redispensed aseptically through a closed system into smaller, sterile, rubber stoppered evacuated bottles. Blood to be used from these bottles should be removed with a sterile syringe and needle. This blood will consist of approximately one part of cells to every three parts of supernate.
- (3) *Serums.* Separate serum from clot and centrifuge until entirely free of cells. Inactivate in water bath (56° C.) for 30 minutes.
- (4) *Spinal fluids.* These are centrifuged before testing, but

they do not contain enough complement to require inactivation by heating at 56° C. Bloody fluids are unsatisfactory for testing since positive results may be due to the activity of the serum rather than that of the spinal fluid. Spinal fluids more than 3 days old, or contaminated with bacteria, may be heated at 56° C. for 15 minutes to destroy the thermolabile anticomplementary substance.

(5) *Egg white.*

- (a) Addition of egg white though not necessary in spinal fluid cardiolipin complement-fixation tests, may be used to lessen the number of anticomplementary reactions.
 - (b) Break a fresh egg and separate the white from the yolk. Pick out any blood spots or other heavy particles. The white may be broken up by forcing it in and out of a 30 ml syringe. An alternate method is to filter through several layers of gauze. Measure the white in a 100 ml graduate and add an equal volume of saline. This 50 percent solution should then be centrifuged in a 50 ml round bottom centrifuge tube at 2,000 rpm for 10 minutes, after which the supernate is decanted into a glass stoppered bottle and placed in the refrigerator. Two-tenths ml is employed in all tubes of the spinal fluid tests. If stored in the refrigerator at 4° to 8° C. this solution can be used for one week before discarding.
- (6) *Other reagents.* Antigen, hemolysin and complement for use in this procedure will be obtained from the Army Medical Service Graduate School, Washington 12, D. C. Cardiolipin complement-fixation antigen should be stored in the dark at room temperature. In an emergency complement may be prepared in the individual laboratory by pooling the fresh sera of at least three normal, healthy guinea pigs which have never been used for any type of biological assay. Pregnant females should not be used. Animals should be given water but no food for 12 hours prior to bleeding. Serum should be stored in the freezing compartment when not in use. Saline to be used for preparation of diluted complement and hemolysin should be brought to refrigerator temperature prior to use. This may be accomplished by placing the required amount of saline for the day's test in the refrigerator during the morning of the day tests are to be run. Complement and hemolysin should be stored in the refrigerator when not in actual use.

c. PREPARATION OF 2 PERCENT SUSPENSION OF SHEEP CORPUSCLES.

- (1) Filter the required quantity of sheep's blood through gauze into a 50 ml centrifuge tube. Add two to three volumes of saline solution. Centrifuge at a speed of 1,800 rpm in a No. 1 International centrifuge (1,580 rpm in a No. 2 centrifuge) for 5 minutes.
- (2) Remove the supernatant fluid and thin upper layer of white cells by suction or a capillary pipette.
- (3) Resuspend the corpuscles in three or four volumes of saline by gentle shaking and again centrifuge. Repeat this procedure three times. The last time, rather than resuspending the corpuscles, they should be washed into a graduated centrifuge tube. If the supernate is not colorless after three washings, the corpuscles are too fragile and should not be used.
- (4) Centrifuge the graduated centrifuge tube containing sheep corpuscles at 2,000 rpm in a No. 1 International (1,700 rpm in a No. 2) for 10 minutes to pack the corpuscles. This speed and time interval should be kept constant from day to day in order to obtain comparable 2 percent suspensions. To prepare the 2 percent suspension, add 49 volumes of chilled saline per volume of *packed* corpuscles. *Do not* prepare the 2 percent suspension by withdrawing and measuring sheep corpuscles with a pipette. Store the 2 percent suspension in the refrigerator at 6° to 8° C. when not in actual use. Always mix the suspension of corpuscles well before using to insure uniformity per unit volume.

d. PREPARATION OF ANTIGEN DILUTION.

- (1) The optimal dilution of cardiolipin complement-fixation antigen has been established at 1:130 (1 ml antigen plus 129 ml Kolmer saline) by repeated titrations with syphilitic sera and spinal fluid. This dilution remains constant because the ratio of cardiolipin: lecithin: cholesterol is fixed in successive lots of antigen; therefore antigen titrations are not indicated and the dilution factor should *not* be altered.
- (2) The required volume of Kolmer saline is first measured accurately into a beaker of suitable size, and the required volume of cardiolipin antigen is then pipetted to the bottom of a second similar beaker. (Not less than 0.2 ml of the cardiolipin antigen should be used in preparing the antigen dilution.) The saline is then poured into the beaker containing antigen as rapidly as possible and the

two are mixed thoroughly by pouring from one beaker to the other six times. The diluted antigen is then ready for use, may be stored either in the refrigerator or at room temperature, and may be used only during the day prepared.

e. TITRATION OF HEMOLYSIN (AMBOCEPTOR).

(1) The hemolysin titration should be performed for each day's tests since age of the corpuscles after bleeding and differences in individual sheep cause a variation in the amount of hemolysin necessary for sensitization.

(2) Prepare a stock solution of 1:100 hemolysin as follows:

Phenol (5 per cent in Kolmer saline solution)	4 ml
Saline solution (Kolmer)	94 ml
Glycerinized hemolysin (50 per cent)	2 ml

Phenol and saline are mixed before addition of glycerinized hemolysin to eliminate the possibility of precipitation of hemolysin by 5 percent phenol. This solution keeps well when stored in the refrigerator, but should be discarded if a precipitate forms.

(3) Prepare a 1:1000 dilution of hemolysin by the addition of 4.5 ml Kolmer saline to 0.5 ml of the stock 1:100 dilution.

(4) In a series of 10 tubes, prepare higher dilutions as in table XLIX.

*Table XLIX. Preparing Dilutions**

0.5 ml hemolysin (1:1,000)	=1:1,000
0.5 ml hemolysin (1:1,000) + 0.5 ml Kolmer saline solution	=1:2,000
0.5 ml hemolysin (1:1,000) + 1.0 ml Kolmer saline solution	=1:3,000
0.5 ml hemolysin (1:1,000) + 1.5 ml Kolmer saline solution	=1:4,000
0.5 ml hemolysin (1:1,000) + 2.0 ml Kolmer saline solution	=1:5,000
0.5 ml hemolysin (1:3,000) + 0.5 ml Kolmer saline solution	=1:6,000
0.5 ml hemolysin (1:4,000) + 0.5 ml Kolmer saline solution	=1:8,000
0.5 ml hemolysin (1:5,000) + 0.5 ml Kolmer saline solution	=1:10,000
0.5 ml hemolysin (1:6,000) + 0.5 ml Kolmer saline solution	=1:12,000
0.5 ml hemolysin (1:8,000) + 0.5 ml Kolmer saline solution	=1:16,000

* Mix the contents of each tube thoroughly.

(5) Prepare a 1:30 dilution of complement for hemolysin and complement titration by diluting 0.2 ml of complement (restored lyophilized or fresh guinea pig serum) with 5.8 ml of chilled Kolmer saline.

(6) In series of 10 tubes set up the hemolysin titration as shown in table L. Reagents should be added in the following order: hemolysin, complement, saline solution, and corpuscles.

(7) Mix the contents of each tube and place them in the water bath at 37° C. for 1 hour. Read the unit of hemolysin. The unit is represented by the tube containing

Table L. Hemolysin Titrations

Hemolysin 0.5 ml of each dilution	Complement (1:30)	Saline solution (Kolmer)	Corpuscles 2 percent
	<i>ml</i>	<i>ml</i>	<i>ml</i>
1:1,000	0.3	1.7	0.5
1:2,000	0.3	1.7	0.5
1:3,000	0.3	1.7	0.5
1:4,000	0.3	1.7	0.5
1:5,000	0.3	1.7	0.5
1:6,000	0.3	1.7	0.5
1:8,000	0.3	1.7	0.5
1:10,000	0.3	1.7	0.5
1:12,000	0.3	1.7	0.5
1:16,000	0.3	1.7	0.5

the highest dilution of hemolysin that gives complete hemolysis. Satisfactory hemolysin should give complete hemolysis with 0.5 ml of a 1:4000 or greater dilution.

- (8) Two units of hemolysin are used in the complement titration and in the complement-fixation tests. Hemolysin is so diluted that two units are contained in 0.5 ml. If the unit is 0.5 ml of the 1:5000 dilution, two units equal 0.5 ml of a 1:2500 dilution. Prepare an amount of the hemolysin dilution indicated which will be sufficient for the complement titration and the day's run of complement-fixation tests.
- (9) Table LI shows how to prepare hemolysin dilutions so that 0.5 ml carries 2 units.

Table LI. Hemolysin Dilutions

1 unit (0.5 ml)	2 units (0.5 ml)	Prepare by diluting 1 ml of 1:100 dilution with the fol- lowing amounts of Kolmer saline solution
		<i>ml</i>
1:4,000	1:2,000	19
1:5,000	1:2,500	24
1:6,000	1:3,000	29
1:8,000	1:4,000	39
1:10,000	1:5,000	49
1:12,000	1:6,000	59
1:16,000	1:8,000	79

- (10) In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration. At the end of the first incubation of the complement titration the unit of hemolysin is available and 2 units are added to all the tubes of the complement titration.

f. TITRATION OF COMPLEMENT.

- (1) The complement titration should be performed at the same time as the hemolytic titration. The same tube of 1:30 complement can then be used for both titrations.
- (2) In a series of 8 test tubes, set up the complement titration as follows:

Table LII. Complement Titrations*

Complement (1:30)	Antigen dilution	Kolmer saline solution	Water bath 37° C. for 1 hour.	Hemolysin (2 units)	Corpuscles (2 percent)	Water bath 37° C. for 1 hour.
<i>ml</i>	<i>ml</i>	<i>ml</i>		<i>ml</i>	<i>ml</i>	
0.20	0.5	1.3		0.5	0.5	
0.25	0.5	1.3		0.5	0.5	
0.30	0.5	1.2		0.5	0.5	
0.35	0.5	1.2		0.5	0.5	
0.40	0.5	1.1		0.5	0.5	
0.45	0.5	1.1		0.5	0.5	
0.50	0.5	1.0		0.5	0.5	
None	None	2.5		None	0.5	

*Add complement, antigen, and Kolmer saline and after 1 hour incubation remove from water bath (37° C.). Then add 2 units of hemolysin and 0.5 ml corpuscles and incubate in water bath (37° C.) for 1 hour before determining the exact unit of complement.

- (3) The smallest amount of complement giving complete hemolysis is the exact unit. The next larger quantity which contains 0.05 ml more complement is the full unit. In conducting the complement-fixation tests employ 2 full units contained in 1 ml as illustrated below:

Exact unit: 0.3 ml
 Full unit: 0.35 ml
 Dose (2 full units): 0.7 ml
 Dilution of complement containing 2 full units
 in 1 ml is $\frac{30}{0.7}$ = dilution 1::43

Table LIII gives additional examples.

- (4) It is always advisable to dilute complement with cold saline solution instead of saline kept at room temperature. Complement, particularly when diluted, should always be kept in a refrigerator when not in use.
- (5) Occasionally hyperactive complement yields a unit of less than 0.3 ml of a 1:30 dilution. If this occurs it is necessary to take arbitrarily 0.3 ml as the exact unit since less complement is likely to be unsatisfactory. If the exact unit is more than 0.45 ml, the complement is unsatisfactory and cannot be used.

Table LIII. Dilution of Complement for the Tests

Exact unit	Full unit	Two full units	Dilution to use	Preparation
<i>ml</i>	<i>ml</i>	<i>ml</i>		
0.30	0.35	0.7	1:43	1 ml complement* + 42 ml Kolmer saline solution.
0.35	0.40	0.8	1:37	1 ml complement + 36 ml Kolmer saline solution.
0.40	0.45	0.9	1:33	1 ml complement + 32 ml Kolmer saline solution.
0.45	0.50	1.0	1:30	1 ml complement + 29 ml Kolmer saline solution.

*Complement refers to the rehydrated lyophilized serum or the fresh guinea pig serum.

g. COMPLEMENT-FIXATION TEST FOR SERUM AND SPINAL FLUID.

- (1) For each serum arrange two test tubes and place 0.5 ml of Kolmer saline solution in No. 2 (control). Add 0.2 ml of inactivated serum to each tube. (See table LIV.)
- (2) For each spinal fluid arrange two test tubes and place 0.5 ml of Kolmer saline solution in No. 2 (control). Add 0.5 ml of spinal fluid to each tube.
- (3) Add 0.5 ml of diluted antigen to the first tube and mix thoroughly.
- (4) Allow to stand at room temperature for 10 to 30 minutes.
- (5) Add 1 ml of complement (2 full units) to all tubes. In the case of spinal fluid tests 0.2 ml of 50 percent egg white may be added to all the tubes including the controls.
- (6) Include the following controls with each series of tests:
 - (a) Antigen control, containing 0.5 ml diluted antigen, 0.5 ml Kolmer saline solution, and 1 ml of complement (2 full units).
 - (b) Hemolytic-system control, containing 1 ml of Kolmer saline solution and 1 ml of diluted complement (2 full units).
 - (c) Corpuscle control, containing 2.5 ml of Kolmer saline solution.
 - (d) Controls of positive and negative sera should be included. (It is advisable to make up a large pool of positive serum and dispense 1 ml in stoppered tubes. These sera may be stored in the freezing compartment of the refrigerator and used as required.)
- (7) Mix the contents of each tube by gentle shaking and place in the refrigerator at 6° to 8° C. for 15 to 18 hours (overnight).

- (8) Place the tubes in a water bath at 37° C. for 10 minutes (not longer).
- (9) To all tubes, except the corpuscle control, add 0.5 ml of hemolysin (2 units), and to all tubes add 0.5 ml of 2 percent corpuscle suspension (well shaken).
- (10) Mix the contents of each tube by thorough shaking of rack and place in the water bath at 37° C. for 10 minutes longer than the time required for the antigen control to clear.
- (11) Table LIV shows the set-up for the complement-fixation test with serum and spinal fluid.

Table LIV. Set up for the Complement-Fixation Tests with Serum and Spinal Fluid

Tube No.		Kolmer saline solution	Antigen	Comple-ment (2 full units)	Refrigerate at 6° to 8° for 15 to 18 hours; then place in 37° C. water bath for 10 minutes.	Hemolysin (2 units)	Corpuscles (2 percent)
		ml	ml	ml		ml	ml
Serum:							
1	0.2 ml -----	None	0.5	1.0		0.5	0.5
2	0.2 ml -----	0.5	None	1.0		0.5	0.5
Spinal fluid:							
1	0.5 ml -----	None	0.5	1.0		0.5	0.5
2	0.5 ml -----	0.5	None	1.0		0.5	0.5
Controls:							
3	Antigen -----	0.5	0.5	1.0		0.5	0.5
4	Hemolytic system --	1.0	None	1.0	0.5	0.5	
5	Corpuscle -----	2.5	None	None	None	0.5	

h. READING AND REPORTING RESULTS.

- (1) It is recommended that reactions in the tests be read by comparison with a series of six standards. Those are prepared daily in test tubes identical with those used for the complement-fixation tests. Diluted intact and hemolyzed sheep's corpuscles are mixed as indicated below in proportions simulating the different degrees of reaction.
 - (a) Diluted sheep's corpuscles are prepared by adding 10.0 ml of standard Kolmer salt solution to 2.0 ml of the standard 2 percent cell suspension.
 - (b) Diluted hemolyzed sheep's corpuscles are prepared by adding 9.5 ml of distilled water to 2.0 ml of the standard 2 percent cell suspension. When lysis is complete, isotonicity is restored by the addition of 0.5

- ml of a concentrated Kolmer salt solution (concentration 20 x) containing sodium chloride 17.0 gm and magnesium sulfate 0.2 gm in 100 ml distilled water.
- (c) Standards for reading complement-fixation tests can be prepared as follows:

Degree of reaction simulated	4 +	3 +	2 +	1 +	±	—
Percent of hemolysis simulated	0	25	50	75	90	100
Diluted hemolyzed sheep's corpuscles, ml.	0	0.75	1.50	2.25	2.70	3.00
Diluted sheep's corpuscles, ml.	3.00	2.25	1.50	0.75	0.30	0

- (2) Standards representing reactions of 4+, 3+ and 2+ are centrifuged for 10 minutes at 2,000 rpm. Corpuscles of the standards representing 1+ and ± reactions are maintained in suspension while in use.
- (3) In reading the results of tests, tubes showing reactions of 1+ or less are read with corpuscles in suspension by comparison with the corresponding standards. Tubes showing stronger reactions are centrifuged for 3 minutes at 2,000 rpm, and read by comparing their supernates with those of the centrifuged standards.** The reaction to be recorded in each instance is that of the nearest corresponding standard.
- (4) Reactions in the tests are reported as positive (4+, 3+, or 2+), doubtful (1+ or ±) or negative (—). If the serum control, containing no antigen, fails to show complete hemolysis the serum is anti-complementary and should be reported as such. A second specimen should be obtained in such cases. If the serum control and the test with antigen show the same degree of partial hemolysis, the serum may safely be reported as negative.
- i. ANALYSIS OF DIFFICULTIES.

- (1) *Due to complement.* In the great majority of instances difficulties are due to complement. Occasionally it is too low in hemolytic activity. Complement deteriorates

**In reading the reactions in centrifuged tubes special attention should be given to any slightly hemolyzed bloods which may have been considered satisfactory for testing. Since the discolored serum may simulate partial lysis of sheep's corpuscles in the centrifuged tests, the amount of color contributed by the serum should be determined by mixing 0.2 ml in a test tube with 2.5 ml of salt solution. If for example, the color of this mixture matches that of the test with antigen, the reaction may be read as 4+.

rapidly at room temperature, especially when diluted. Complement should always be diluted with cold saline and kept in the refrigerator at *all* times except when in use. Complement is not fixed non-specifically with cardiolipin as it sometimes is with Kolmer antigen. Consequently it is unnecessary, in tests with spinal fluid, to use egg white in order to minimize false positive reactions. The practice may be retained, however, as a means of reducing the incidence of anticomplementary spinal fluid reactions.

- (2) *Due to hemolysin.* This is usually the first reagent suspected, but least likely to be a cause of trouble, especially if it has been previously used with success. If the saline solution and complement are satisfactory, a good hemolysin rarely causes difficulty even after being shipped long distances. If no other source of trouble is found, verify titer of hemolysin.
- (3) *Due to corpuscles.* When blood is obtained from a slaughterhouse one sooner or later encounters specimens that have an increased resistance to serum hemolysis. Such blood must be discarded.
- (4) *Due to antigen.* This is rarely a cause of trouble if no mistakes have occurred in dilution and dosage. When the antigen control shows incomplete hemolysis it is usually due to some component of the hemolytic system, most likely the complement.
- (5) *Due to saline.* If the saline has been properly prepared no trouble should be encountered. If through error in weighing of NaCl or addition of distilled water a hypotonic solution is prepared, the corpuscles may show slight hemolysis on washing though they appeared satisfactory on the first centrifugation. If similarly a hypertonic solution is prepared, difficulty may be experienced when the titrations are performed, since the corpuscles may not completely hemolyze. In such cases the complement will probably be suspected of being defective. In humid climates it is well to dry the NaCl in a hot air oven (160–200° C.) for 15 or 20 minutes, cooling to room temperature and then weighing to insure an isotonic solution.
- (6) *Due to anticomplementary serums and spinal fluids.* Serums and spinal fluids may be found to be anticomplementary, as shown by incomplete or no hemolysis in the controls. After experience has been gained some of the partial reactions may be read with safety as doubtful or

negative, but as a general rule it is safer and wiser to repeat the tests with fresh serum or spinal fluid.

Caution: Tests sometimes have to be repeated, and for this reason the unused portions of all serums and spinal fluids should be routinely kept in a refrigerator until the tests are completed.

445. Cardiolipin Microflocculation Test

a. GLASSWARE AND APPARATUS.

- (1) *Pipettes.* The following pipettes are recommended:
 - 1 ml, graduated in 0.01 ml (to tip)
 - 5 ml, graduated in 0.10 ml (to tip)
 - 10 ml, graduated in 0.10 ml (to tip)
- (2) *Antigen dilution bottle.* 30 ml, round, narrow mouth glass stoppered bottle.
- (3) *Syringe.* 1 ml Luer tuberculin syringe.
- (4) *Needle.* 23 gauge, $\frac{3}{4}$ inch hypodermic needle.
- (5) *Slides.* Boerner, 10 cell, glass, micro slide.
- (6) *Rotating apparatus.* Slide rotating apparatus.
- (7) *Water bath.* Any easily regulated water bath that can be used satisfactorily at 56° C. is suitable.
- (8) *Microscope, monocular.*

b. REAGENTS.

- (1) Cardiolipin microflocculation antigen will be obtained from the Army Medical Service Graduate School, Washington 12, D. C. It should be stored in the dark at room temperature. No antigen from other sources will be employed.
- (2) Buffered antigen diluent for the preparation of the antigen emulsion will be obtained from the Army Medical Service Graduate School, Washington 12, D. C. It should be stored at room temperature. The buffered diluent is supplied ready for use. The pH should be checked frequently and any buffered diluent found to be outside the range pH 5.9 to 6.1 should be discarded.
- (3) Clear serum obtained by centrifuging whole clotted blood is heated for 30 minutes in the water bath at 56° C. before testing. All serums are examined upon removal from the water bath and those found to contain small particles are clarified by recentrifugation. Serums to be tested more than 4 hours after heating should be reheated for 10 minutes in the water bath at 56° C.

c. CLEANING OF GLASS SLIDES.

- (1) New slides are first cleaned with sulfuric and chromic acid mixture (see par. 21a). They are then rinsed

repeatedly in hot running tap water to remove all traces of the cleaning solution, and are rinsed finally in distilled water. Used slides are immersed in a pan of tap water immediately after the tests have been read in order to prevent drying of the serum-antigen mixture. As soon as possible they are washed carefully with soap and water, using a soft bristle brush, and are then freed of soap by repeated rinsings with hot tap water and distilled water as above. All slides in continual use should be cleaned at least once weekly with the sulfuric and chromic acid mixture. Bon Ami should not be used since it is difficult to remove from the moats surrounding the slide cells.

- (2) Cleaned slides are inverted and allowed to drain on a *clean* hand towel. They may then be wiped dry with a *clean* soft cloth, and are stored on edge in a clean, dust-proof box. Slides are handled by the edges while cleaning to prevent greasy finger prints on the testing surfaces. Serum will spread within the cells on clean slides. Failure to spread indicates that the slide is unclean and should not be used.

d. PREPARATION OF ANTIGEN EMULSION.

- (1) Antigen emulsion is prepared by pipetting 0.4 ml of the buffered antigen diluent to the bottom of a 30 ml round glass bottle fitted with a glass stopper. While the bottle is being rotated gently and continuously on a flat surface, 0.5 ml of the antigen is added drop by drop (rapidly, without long intervals between drops) from the lower half of a 1.0 ml pipette that is graduated to the tip. During this operation the pipette tip should remain in the upper third of the mixing bottle, antigen should be dropped directly into the diluent, and rotation should not be so vigorous as to splash diluent on the pipette tip. The last drop of antigen is blown from the pipette without touching it to the diluent, and rotation is continued for 10 seconds more. Using a 5.0 ml pipete, 4.1 ml of antigen diluent is then added, the top is placed on the bottle, and the latter is shaken vigorously for 10 seconds (throwing the liquid from the bottom to the top of the bottle). The antigen emulsion is then ready for use and may be used during one day if stored at refrigerator or room temperature. This amount of antigen (5.0 ml) is sufficient for approximately 250 qualitative serum tests.
- (2) Twice this amount of antigen emulsion may be prepared at one time in a 30 ml bottle by using doubled quantities of antigen and antigen diluent. A 10.0 ml pipette should

then be used for delivering the required 8.2 ml of diluent. If larger quantities of antigen are required more than one mixture should be prepared and the emulsions then pooled and tested. (See *f* below.)

- (3) Under conditions of high temperatures and low humidity such as are encountered during the summer months in certain areas, antigen emulsion may be stored in the refrigerator. To minimize the effects of evaporation in tests carried out under these conditions, the tests should be completed and read as rapidly as possible.

e. PRETESTING OF ANTIGEN NEEDLE: DELIVERY OF ANTIGEN EMULSION.

- (1) Since the number of antigen particles per microscopic field is dependent upon the size of the antigen emulsion drop, the delivery needle used each day should be checked.
- (2) Antigen emulsion is delivered from a 23 gauge, long bevel, hypodermic needle attached to a 1.0 ml syringe, which is allowed to stand in the antigen emulsion bottle when not in use. About 60 drops should be obtained from 1 ml of antigen emulsion. This can be accomplished by holding the syringe so that the needle bevel faces downward and its dropping surface is horizontal. Increasing the angle at which the syringe is held decreases the dropping surface and consequently reduces drop size. Practice will allow rapid delivery of antigen emulsion, but care must be taken to obtain drops of equal size. When the antigen emulsion has been allowed to stand it should be gently mixed before use by rotating the bottle and by filling and emptying the syringe.

f. PRETESTING OF ANTIGEN EMULSION. Each preparation of antigen emulsion should first be examined in preliminary tests of known positive and negative control serums. The technic used is that given for the qualitative serum test. These preliminary tests should show typical positive and negative results respectively, and the number of antigen particles per microscopic field in the test with negative serum should be optimal.

g. TECHNIC OF QUALITATIVE TEST.

- (1) Pipette a 0.05 ml volume of 10 clear heated serums each into its respective ring on a glass slide.
- (2) Add one drop (approximately $\frac{1}{60}$ th ml) of antigen emulsion to each serum.
- (3) Rotate the slides for 4 minutes on a Boerner type electric rotating machine at 180 rotations per minute. The speed of rotation should be checked carefully and the

time of 4 minutes adhered to strictly, since variation will vary the sensitivity of the tests.

(4) Read the tests immediately after rotation.

h. READING AND REPORTING RESULTS.

(1) Proper reading and interpretation of test results requires that the technicians have had adequate training and experience with the given technics.

(2) The tests are read microscopically with low power objective at 100 X magnification. The antigen particles in properly prepared emulsions appear as short rod forms. Aggregation of the particles in small, medium, or large clumps is translated into degree of reaction and reported as follows:

Appearance of tests	Degree of reaction	Report
No clumping or very slight roughness...	—	Negative
Small clumps.....	± or 1 +	Doubtful
Medium or large clumps.....	2 +, 3 +, or 4 +	Positive

(3) Zonal reactions (reactions less than 4+ occurring in serums which upon dilution yield stronger reactions) are recognized by atypical or feathery clumping and the loosely bound characteristics of the clumps. The usual positive reaction is characterized by large or small clumps of fairly uniform size, and experience will permit the differentiation of this usual type of reaction from the zonal reactions in which large and/or small clumps are intermingled with free antigen particles. In instances where a zonal type of reaction is observed or suspected, the particular serum should be subjected to the quantitative serum test. If the serum gives a positive reaction on dilution it should be reported *Positive*.

446. Quantitative Cardiolipin Microflocculation Test

a. TECHNIC. Quantitative tests are performed with serial dilutions of serum in salt solution. Each dilution is treated as an individual serum. Serial two-fold dilutions of serum are prepared by measuring 0.5 ml of freshly prepared 0.9 percent sodium chloride solution into each of 6 or more test tubes. Five-tenths ml of heated serum is added to tube 1, mixed well, and 0.5 ml of the diluted serum transferred to tube 2. This operation is continued through the successive tubes until tube 6 contains 1.0 ml of diluted serum. The resulting serum dilutions are 1:2, 1:4, 1:8,

1:16, 1:32, and 1:64. Each serum dilution is tested as in the qualitative serum test.

b. **REPORTING RESULTS.** The greatest dilution yielding a positive (2+, 3+, or 4+) reaction is reported as the serum titer. The following are examples of the calculation of serum titer; serums A, B, and C are to be considered as having given typical positive reactions in the qualitative test, serum D as having given an atypical, zonal reaction (less than 4+) in the qualitative test.

	Reaction with serum diluted						Report
	1:2	1:4	1:8	1:16	1:32	1:64	
Serum A.....	4	4	4	2	±	—	Positive-Titer 16
Serum B.....	4	3	1	—	—	—	Positive-Titer 4
Serum C.....	1	—	—	—	—	—	Positive-Titer 1
Serum D.....	3	4	4	4	2	—	Positive-Titer 32

447. Colloidal Gold Test with Spinal Fluid

a. APPARATUS AND GLASSWARE.

(1) *Pipettes.*

1 ml graduated in 0.01 ml (to tip).

10 ml graduated in 0.10 ml (to tip).

(2) *Test tubes.* Heat resistant test tube (approximately 15 mm. outside diameter) for colloidal gold test should be kept separate from all others used in the laboratory. Cleaning is accomplished with aqua regia.

(3) *Cylinders, graduated.*

1,000 ml

100 ml

50 ml.

(4) *Flasks.*

2,000 ml Erlenmeyer, heat resistant.

(5) *Beakers.*

250 ml.

(6) *Burner, Bunsen.*

High temperature.

(7) *Bottles.*

Narrow mouth, round amber, 120 ml.

Narrow mouth, round, 1 liter.

(8) *Thermometer.*

Minus 10 to plus 110° C.

b. REAGENTS.

(1) *Double distilled water*, last distillation from glass still.

- (2) *Gold chloride, U.S.P. 1.0 percent.* The label should be soaked off a 15 grain ampule, and the ampule thoroughly rinsed with double distilled water. Break and place the ampule and contents into a beaker containing 97 ml of double distilled water. Store the 1 percent solution of gold chloride in a 100 ml glass stoppered amber bottle.
- (3) *Sodium citrate, U.S.P., 1.0 percent.* One percent sodium citrate is prepared by weighing out 5 grams of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and dissolving in a sufficient quantity of double distilled water to make 500 ml of solution. The solution must be freshly prepared.
- (4) *Hydrogen peroxide, U.S.P., (3 percent).* Use a freshly opened bottle.
- (5) *Sodium chloride, A.C.S., 0.4 percent.* Prepare a 0.4 percent solution by dissolving 4 grams of sodium chloride in sufficient double distilled water to make 1 liter of solution.
- (6) *Stock globulin solution.* Ten grams of jack bean meal are extracted with 40 ml of 1.0 percent sodium chloride solution for 30 minutes with shaking at frequent intervals. This mixture is passed through a Seitz filter containing a previously washed No. 1 serum pad. The filtrate is centrifuged at high speed, and the supernate is added to an equal amount of chemically pure, neutral glycerin. This stock solution will retain its reactivity for a period of nine months when stored at 6° to 8° C. It may be used in lieu of a zone 1 spinal fluid as a colloidal gold control. Stock globulin solution is requisitioned from the Army Medical Service Graduate School, Washington 12, D. C.

c. PREPARATION OF STOCK GOLD SOLUTION.

- (1) Pipette 10 ml of the 1.0 percent gold chloride solution into 950 ml of double distilled water in a 2000 ml heat resistant Erlenmeyer flask.
- (2) Bring the solution as rapidly as possible to between 90° and 95° C. Remove the thermometer when the solution reaches 92° C.
- (3) Immediately add 50 ml of 1.0 percent sodium citrate solution without removal of the flask from the flame.
- (4) When the solution comes to a boil quickly add 0.77 ml of hydrogen peroxide. Care should be taken to exclude the many small bubbles that form in a freshly opened bottle. With the addition of the hydrogen peroxide there should be an immediate color change.

- (5) Do not boil the solution after addition of hydrogen peroxide. Remove from the flame and cool.
- (6) *The flask should not be shaken at any time during the procedure.*
- (7) When the colloidal gold solution has cooled, pour it into a 1 liter, heat resistant, glass stoppered bottle and store in the dark at room temperature.
- (8) Approximately 10 minutes are required to prepare the colloidal gold when a hot flame is used.
- (9) There is no bluish tinge to the colloidal gold; its color is a burgundy red in the 2 liter flask when viewed against the light of a window.
- (10) Formation of a blue precipitate indicates that the colloidal gold is no longer usable.

d. TEST PROCEDURE.

- (1) Place 0.9 ml of freshly prepared 0.4 percent sodium chloride solution in the first tube and 0.5 ml in each of the other nine tubes.
- (2) To the first tube add 0.1 ml of the spinal fluid to be tested and mix thoroughly.
- (3) Remove 0.5 ml of the mixture from the first tube and place in the second tube, mixing thoroughly as before.
- (4) Remove 0.5 ml from the second tube and place in the third tube, continuing these transfers until the tenth tube is reached, from which 0.5 ml is discarded.
- (5) Add 2.5 ml of the colloidal gold solution.
- (6) Mix contents of tubes and allow to remain at room temperature, in the dark, for 18 to 24 hours.
- (7) Previously tested spinal fluids that give negative and zone 1 curves should be included with each group of specimens tested. If a satisfactory zone 1 spinal fluid is not available, a standardized globulin solution may be used.
- (8) The following method is used to prepare a 1:60 dilution of globulin solution when no known strongly positive spinal fluid is available. The globulin solution is removed from the stock bottle with a 0.1 ml pipette and diluted with 5.9 ml of 0.4 percent sodium chloride solution. The required amount is drawn up into the pipette and, after wiping the outside to remove the excess globulin solution, is blown into the measured volume of 0.4 percent sodium chloride. The pipette should be thoroughly rinsed to remove all the globulin solution by drawing saline into

the pipette at least 10 times. The solution is mixed well and then employed in the same manner as spinal fluid. It is important that no variation be made in the above technic, since greater or lesser amounts of globulin solution will change the appearance of the zone 1 type curve.

- (9) One control tube containing 0.5 ml of 0.4 percent sodium chloride solution and 2.5 ml colloidal gold solution should also be included in each series of tests.

e. REPORTING RESULTS.

- (1) Color changes occurring in each tube are recorded numerically in accordance with the following. The tube containing only salt solution and colloidal gold served as a negative control:

<i>Number</i>	<i>Color Change</i>
0	Unchanged, as compared with the control.
1	Reddish purple.
2	Purple.
3	Blue.
4	Light pink or blue (almost decolorized).
5	Clear (completely decolorized).

- (2) The following readings are representative of the four common types of colloidal gold reactions:

Zone 1:	5 5	5 5	5 5	5 4	5 3	4 2	2 1	1 0	0 0	0 0
Zone 2:	0 0	1 1	2 2	3 3	2 4	1 2	0 1	0 0	0 0	0 0
Zone 3:	0 0	0 0	0 1	1 2	2 3	3 4	4 5	2 3	1 1	0 0
Negative:	0 1	0 1	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

- (3) The shape of the curve is of no diagnostic significance. The last tube showing a definite color change is a rough measure of the globulin content of the fluid.

Note. Spinal fluids containing blood or gross bacterial contamination cannot be expected to yield the same type of colloidal gold reaction that would be obtained if these contaminating substances were not present. Relatively small amounts of blood may contribute enough serum to reduce or eliminate a zone 1 or 2 colloidal gold curve. The effect of gross bacterial contamination of spinal fluid on the colloidal gold reaction is unpredictable; for this reason such fluids should be considered unsatisfactory for this test.

CHAPTER 13

PROTOZOOLOGICAL METHODS

Section I. PROTOZOA

448. General

a. The protozoa are single-celled animals of microscopic size. They are important because some species of protozoa inhabit various parts of the human body and cause disease. Amebic dysentery, malaria, and African sleeping sickness are three diseases caused by protozoa.

b. The protozoa are larger than bacteria and their internal structure is more easily observed. This structure is usually diagnostic of the species. Many parasitic protozoa can be grown quite readily, and others with difficulty on artificial media, whereas still others cannot yet be thus cultivated.

c. Although protozoal diseases are widespread in tropical countries, some occur also frequently in temperate zones. They are of importance because of their epidemic tendencies and because of the fact that a large number of military personnel may have served, or are now serving, in areas where they are common. Many men may develop chronic or asymptomatic infections, and cases of disease and, acting as carriers, serve to infect others.

Section II. INTESTINAL PROTOZOA

449. General

a. AMEBAE. Included among the protozoal parasites of man are the amebae, which move about by pseudopodia (false feet). Of

1-9, *Endamoeba histolytica*: 1 and 5, uninucleate cyst with diffusely stained glycogen vacuole; 2 uninucleate cyst with chromatoid bar; 3, 6, 7, and 9, binucleate cysts (6, with diffuse glycogen, and 9, with chromatoid bar); 4 and 8, quadrinucleate cysts. 10-13, *Endolimax nana*: 10, quadrinucleate cyst; 11 and 12, binucleate cyst, (11, with stained glycogen mass); 13, Uninucleate state. 14-19, *Endamoeba coli*: 14, quadrinucleate stage; 15 and 16, binucleate stage (15, with deeply stained glycogen mass); (17, 18 and 19, with concentrated nuclei). 20-21, *Giardia lamblia*. 22-23, *Chilomastix mesnili*: 23, with deeply stained glycogen (rare). 24, *Iodamoeba butschlii* showing characteristic deeply stained, sharply delimited, glycogen mass. 25, *Blastocystis hominis* (a common vegetable form with cyst-like aspect).

Figure 30

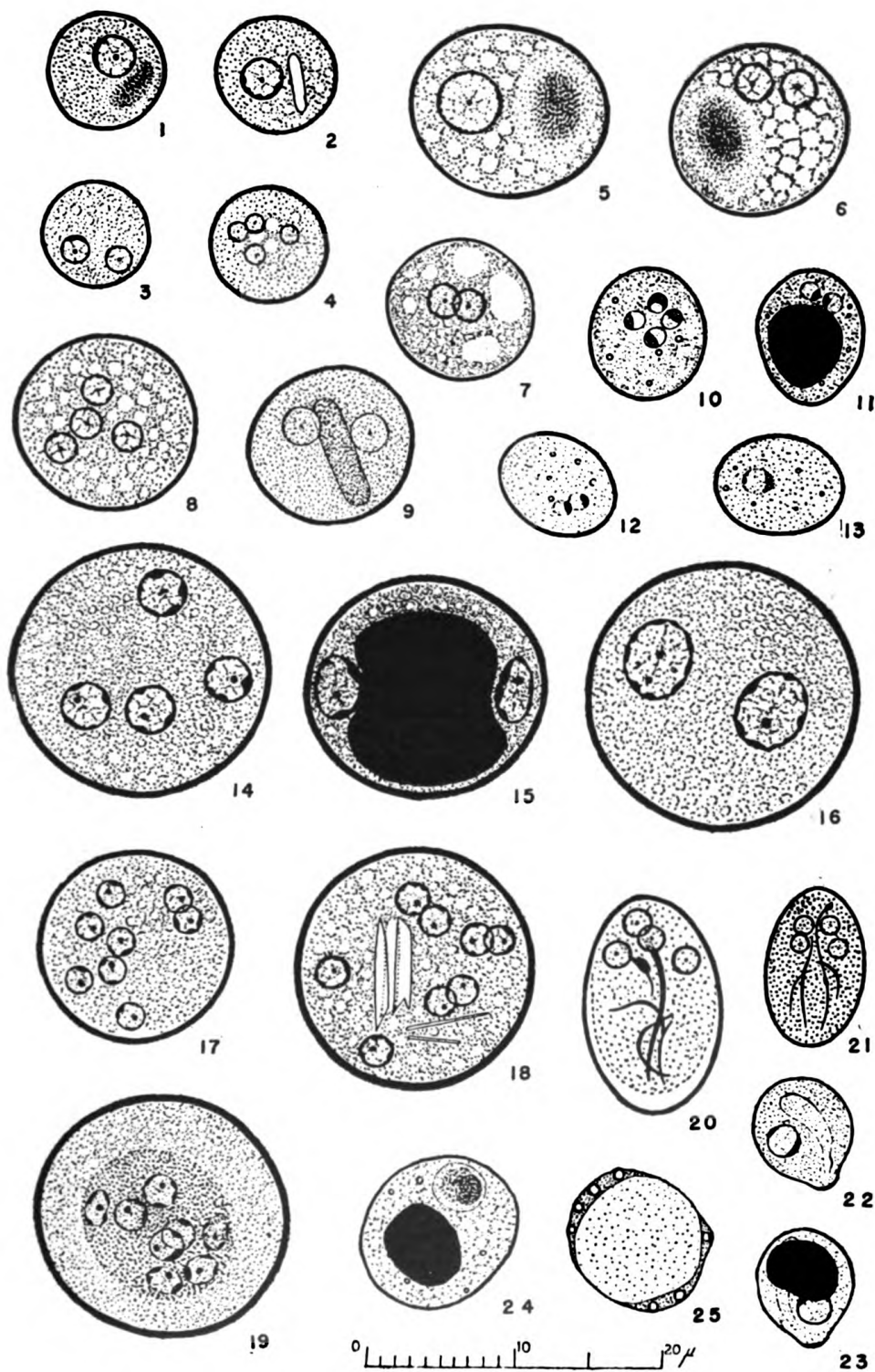


Figure 30. Cysts of intestinal protozoa of man stained with iodine.

Table LV. Characteristics of Common Amebae of Man

Characteristic	<i>Endamoeba histolytica</i>	<i>Endamoeba coli</i>	<i>Endolimax nana</i>	<i>Iodamoeba butschlii</i>	<i>Dientamoeba fragilis</i>
TROPHOZOITES:					
Size-----	18-25 mu-----	20-30 mu-----	8-10 mu-----	10-20 mu-----	3.5-12 mu.
Motility (fresh)-----	Active, progressive, streaming.	Sluggish, seldom progressive.	Active, progressive.	Sluggish, progressive.	Active; clear pseudopods preceding granular mass.
Motility (cool)-----	Suddenly extended clear, sharply defined, pseudopods.	Slowly extended, not sharply defined, pseudopods.	Short, blunt clear, sluggish pseudopods	Sluggish pseudopods	Inactive (soon degenerates).
Cytoplasm-----	Finely granular, non-vacuolated and greenish; ingested red cells diagnostic.	Coarsely granular; vacuolated and grayish; bacteria, yeast, etc., included.	Clear and finely granular; bacterial inclusions.	Finely granular and grayish; bacterial inclusions.	Endoplasm vacuolated; ectoplasm clear; colorless; bacterial inclusions.
Nucleus-----	Fresh: invisible. Stained: fine membrane, beading and delicate, usually central karyosome.	Fresh: visible as ring. Stained: coarser membrane, coarse beading and large and usually eccentric karyosome.	Fresh: karyosome faintly visible. Stained: no beading; karyosome, with one, two, or three masses, each central or eccentric.	Fresh: karyosome visible. Stained: no beading; large, single, deeply stained karyosome.	Fresh: inconspicuous. Stained: generally two nuclei; no beading; karyosome consists of a group of chromatin grains.

CYSTS:					
Size, shape.....	Roundish, 7-18 mu.	Roundish, 10-25 mu.	Ovoidal, 5-14 mu.	Irregular, 8-15 mu.	No cysts.
Nuclei, number.....	Young, 1 or 2; mature, 4.	Young, 1, 2, 4; mature, 8.	Young, 1 or 2; mature, 4.	Mature 1.....	No cysts.
Nuclei, structure.....	As in trophs.....	As in trophs.....	As in trophs.....	Eccentric karyosome beside granular mass.	No cysts.
Chromatoid bodies.....	Thick or slender bars; often absent.	Inconstant. Like glass splinters or irregular.	No true chromatoids.	No true chromatoids.	No cysts.
Glycogen mass (in iodine).	Diffuse, brown. In young cysts.	Large, deep brown. In young cysts.	Occasionally present in young cysts.	Large or small sharply delimited, deep brown.	No cysts.

the species inhabiting the intestinal tract of man, only one, *Endamoeba histolytica*, is pathogenic. Although amebic dysentery (due to *E. histolytica*) is of more common occurrence in tropical and subtropical regions, it has at times reached epidemic proportions in temperate climates. It is estimated that in the United States alone 6,750,000 to 13,500,000 persons are carriers of this parasite, an incidence of 5 to 10 percent. Accurate diagnosis of *E. histolytica* is absolutely essential. An erroneous diagnosis, based on the finding of one of the harmless ameba, subjects the patient to needless treatment. The intestinal ameba most commonly confused with *E. histolytica* is *E. coli*. Table LV and figure 30 will enable the laboratory worker to differentiate these and several other species of amebae.

b. OTHER INTESTINAL PROTOZOA.

- (1) Other intestinal protozoa include such flagellates as *Chilomastix mesnili*, *Trichomonas hominis*, and *Giardia lamblia*. These organisms are probably harmless, although there is some evidence that *G. lamblia* is sometimes pathogenic.
- (2) *Balantidium coli* is an intestinal ciliate of man sometimes producing a dysenteric condition similar to that caused by *E. histolytica*. Further details regarding all these forms may be found in standard texts.

450. Collections of Specimens

a. Stools should be collected directly into a clean receptacle fitted with a cover (bedpan). If a suitable container is not available, a newspaper placed over the toilet seat will serve. With the regulation metal spoon designed for this purpose, a small amount of the feces is placed in a small glass vial, which is then stoppered. Care should be taken to prevent mixing urine with stool. No preservative is used.

b. All stool specimens should be examined at the earliest possible moment, as the protozoa rapidly disintegrate.

c. Fecal specimens should be free of oil and barium, since these substances render stools practically useless for examination for protozoa.

d. If the pathologic lesions are in the rectum or sigmoid, specimens may be obtained by the physician by means of the proctoscope or the sigmoidoscope and sent to the laboratory for examination. These are more apt to yield *E. histolytica* than the passed feces. However, because of the attending discomfort to the patient, this method should be used only after finding the feces negative.

e. Saline purgation and examination of the resulting liquid stools for vegetative forms (trophozoites) is a method commonly employed to detect amebic infection. Formed stools usually contain only cysts of protozoa, whereas semifformed or liquid stools will ordinarily contain the trophozoites.

f. Protozoa may be present in great numbers one day and relatively scarce the next. Therefore, to arrive at a satisfactory diagnosis in questionable cases, repeated examinations should be made on 3 to 8 consecutive days, if feasible.

451. Examination of Fresh Material

The routine examination of fecal specimens for intestinal protozoa should include, as a minimum, three direct wet smears, with unstained and iodine-stained halves, and a preparation using the zinc sulfate flotation technic.

a. **DIRECT WET SMEAR.** This is a two-cover-slip preparation made directly from the stool specimen. A drop of physiologic saline solution and a drop of D'Antoni's iodine stain or Lugol's solution (*e* and *f* below) are placed separately, about 25 millimeters apart, on a clean glass slide (75 by 38 millimeters, if available). If the stool contains blood or mucus, this portion should be selected for making the smears. A small quantity of the material is spread evenly with a wooden applicator or a toothpick, first in the drop of saline and then in the iodine stain. The proper density of the smears should be such that newspaper print when viewed through the preparations is still legible. Cover slips are applied to the two smears, and the preparations are examined with the high dry objective. The organisms are easily detected on the unstained side; however the iodine-stained portion usually must be referred to for identification of cysts, on the basis of morphologic detail. The diagnostic chromatoid bodies inside *E. histolytica* cysts, however, usually show better in the unstained preparation.

b. **ZINC SULFATE CENTRIFUGAL FLOTATION TECHNIC.** In formed or semifformed stools where cysts are likely to be found, the zinc sulfate centrifugal flotation technic is valuable. Ordinary fecal smears should also be made, however, since they give some indication of the intensity of the infection, the zinc sulfate method having its greatest value in enabling the worker to discover small numbers of protozoa. The method is as follows:

- (1) Prepare a fecal suspension by emulsifying 1 part of stool specimen (about the size of a thumbnail) in 10 parts of lukewarm water.

- (2) If the stool contains large quantities of coarse material, strain 10 ml of this emulsion through two layers of wet cheesecloth into a Wassermann tube.
- (3) Centrifuge for 45 to 60 seconds at approximately 2,500 rpm, pour off the supernatant fluid, and add 2 to 3 ml of water. Then break up the sediment, fill the tube with tap water, and repeat the above, centrifuging and discarding the supernatant fluid two to four times, or until the supernatant fluid is clear.
- (4) After pouring off the last supernatant fluid, add zinc-sulfate solution to fill the tube to about 12 millimeters from the rim. (Zinc-sulfate solution is made up by dissolving 331 gm zinc sulfate— $\text{ZnSO}_4 + 7\text{H}_2\text{O}$, granular U.S.P.—in sufficient distilled water to make 1 liter of solution, or 371 gm in 1 liter of water. In either event the specific gravity should be adjusted after 24 to 48 hours to exactly 1.180 and regularly checked before use with a suitable hydrometer.)
- (5) Centrifuge the tube for 45 to 60 seconds at top speed (3,000 rpm).
- (6) Remove several loopfuls of the material floating on the surface film to a clean slide; add 1 drop of iodine stain and a cover slip.

c. SIMPLIFIED FLOTATION TECHNIC. If a centrifuge is not available, cysts may be concentrated as outlined in (1), (2), and (3) below. However, the final preparation is not so clear and the enrichment of cysts is not so great as with the original technic. The procedure for this modification is as follows:

- (1) A portion of stool the size of a pea is introduced into a glass vial (the standard type used for collecting fecal specimens).
- (2) About 2 ml of sugar or salt solution, specific gravity 1.180 (par. 473), is added and the feces thoroughly emulsified with an applicator; more solution is added to bring the level of the liquid to the top of the tube, the larger particles being removed from the surface with an applicator.
- (3) A cover slip (20 by 20 millimeters) is placed on the top of the vial in contact with the surface film and allowed to stand for 20 to 30 minutes, at the end of which time the cover slip is lifted off and placed, wet side down, on a slide containing a drop of iodine stain.

d. AQUEOUS SOLUTION. When *Blastocystis hominis*, a vegetable organism, is present in such quantity as to obscure amebic cysts,

the former may be destroyed by substituting tap or distilled water for the physiologic saline solution in preparing a fresh mount. Vegetative amebae and motile flagellates are also destroyed by this procedure.

e. D'ANTONI'S STANDARDIZED IODINE STAIN. To prepare the staining solution, add to 100 ml of a 1-percent solution of potassium iodide (obtained by dilution of an accurately prepared 10-percent solution), 1.5 gm of powdered iodine crystals. This solution must stand for 4 days before it is ready for use. It should be filtered before using, and the container should not be allowed to remain unstoppered, since volatilization of the iodine will occur. The stock solution, if tightly stoppered, can be kept for long periods without deterioration.

f. LUGOL'S IODINE SOLUTION. This is quite satisfactory for staining protozoal cysts. It is 5 gm iodine and 10 gm potassium iodide and 100 ml of distilled water.

452. Preparing Fecal Smears For Permanent Mounts

The stool should be fresh, with the protozoa showing no evidence of degeneration. There is practically no advantage in staining fecal material in which no organisms can be found by the examination of fresh preparations.

a. PREPARATION AND FIXATION OF SMEAR. A thin fecal smear is made with an applicator on a clean slide. Without allowing the smear to dry, place it with smear side down into warm Schaudinn's fluid (56° C.) for 12 minutes. Remove slide to jar containing cold Schaudinn's to remain for 20 minutes. The fixing solution is prepared as follows:

Mercuric chloride (saturated solution in distilled water)	65 ml
Ethyl alcohol (95 per cent)	35 ml
Glacial acetic acid	5 ml

b. STAINING OF SMEAR.

(1) *Procedure.* The fixed specimens prepared as outlined above are carried through the successive steps in staining as follows:

- (a) Immerse for 10 minutes in 70 percent ethyl alcohol.
- (b) Immerse for 10 minutes in 70 percent ethyl alcohol to which has been added sufficient iodine stain to produce a light mahogany color.
- (c) Immerse for 10 minutes in 70 percent ethyl alcohol.
- (d) Mordant in the following solution for 20 minutes:

Iron alum (ferric ammonium sulfate) 4 per cent aqueous solution	1 part
Ethyl alcohol (50 per cent)	10 parts

- (e) Immerse for 5 minutes in 70 percent ethyl alcohol.
- (f) Stain for 24 hours in Heidenhain's iron hematoxylin, prepared as follows:

Hematoxylin	1 gm
Ethyl alcohol (95 per cent)	10 ml
Distilled water	90 ml
Thymol	1 crystal

Dissolve the hematoxylin in the alcohol, add the distilled water and thymol, then allow the solution to ripen for 1 month in a clear, glass-stoppered bottle exposed to the sun.

- (g) Wash in two changes of tap water.
- (h) Decolorize in the following solution:

Iron alum (2 per cent aqueous solution)	1 part
Ethyl alcohol (50 per cent)	10 parts

Differentiate by agitating each slide separately in the above solution until a light gray to blue tinge predominates; control the exact point by observing the staining definition of the organism under the microscope every few minutes.

- (i) Rinse in tap water, then wash for 10 minutes each in three separate dishes of 40, 60, and 80 percent ethyl alcohol.
- (j) Begin dehydration by two changes of 95 percent ethyl alcohol for 5 minutes each.
- (k) Complete dehydration by two changes of absolute ethyl alcohol for 10 minutes each.
- (l) Replace the absolute alcohol in the specimen by two changes of xylol for 10 minutes each.
- (m) Mount in canada balsam or Clarite.
- (n) Examine under oil immersion.

(2) *Precautions.*

- (a) The specimen should not be allowed to dry at any stage in the technic, since drying causes the organisms to shrink and become distorted in shape.
- (b) If the decolorizing agent is not thoroughly washed out of the specimen, it will fade the stain.
- (c) If the specimen is not properly dehydrated before clearing in xylol, the xylol will become milky and the slide, when viewed microscopically after mounting in balsam, will appear blurred.

c. RAPID METHOD OF FIXING AND STAINING SMEARS (Markey, Cubbertson, and Giordano: Am. J. Clin. Path. 13 1943).

- (1) Fix smears in Schaudinn's fluid for 2 minutes or more.
- (2) Immerse in 95 percent ethyl alcohol (with iodine, if permanent mounts are desired) for 15 to 30 seconds.
- (3) Rinse in water.
- (4) Mordant for 2 to 3 minutes at 56° C. in the following solution:

Ferric ammonium sulphate	5 gm
Distilled water	100 ml

- (5) Rinse in water.
- (6) Stain for 1 to 2 minutes at 56° C. in the following hematoxylin solution:

Hematoxylin (10 per cent alcoholic solution)	0.4 ml
Glacial acetic acid	0.8 ml
Distilled water	40.0 ml

- (7) Rinse in water and allow to stand until blue black (for permanent mounts, to prevent fading, slides should be washed in running water for 15 to 30 minutes).
- (8) Dehydrate in 95 percent ethyl alcohol for 30 seconds to 1 minute, followed by absolute ethyl alcohol or acetone for 1 minute.
- (9) Clear in xylol.
- (10) Mount in Clarite or canada balsam.
- (11) Examine under oil immersion.

d. A RAPID STAIN TECHNIC FOR INTESTINAL PROTOZOA USING TERGITOL-HEMATOXYLIN (Diamond: Am. J. Clin. Path. 15.68, 1945.)

- (1) Emulsify a small bit of representative feces about the size of a pea in physiological salt solution. Avoid making emulsion too fluid or too viscous.
- (2) With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide or cover-slip. Care should be taken not to allow the smear to dry. If drying does occur at any step in the procedure, discard the smear.
- (3) Fix immediately in Schaudinn's sublimate alcohol, which has been heated to 60° C. and to which 5 percent glacial acetic acid has been added just prior to use—2 minutes.
- (4) 50 percent alcohol—1 minute.
- (5) Distilled water—1 minute.
- (6) Mordant in 4 percent ferric ammonium sulfate—5 minutes.
- (7) Rinse quickly in distilled water.
- (8) Stain 5 minutes in tergitol-hematoxylin.

- (9) Rinse in distilled water.
- (10) Destain in saturated aqueous picric acid solution—3 to 5 minutes, depending upon thickness of smear.
- (11) Rinse in 2 changes of distilled water—30 seconds each.
- (12) Set stain by blueing in 70 percent alcohol to which has been added a few milligrams of potassium acetate to raise pH above 7. Bromthymol blue or phenol red may be used as an indicator.
- (13) 70 percent alcohol—1 minute.
- (14) 95 percent alcohol—1 minute.
- (15) Absolute alcohol or a clearing agent such as carbonyxylol. (Xylol saturated with phenol crystals.)
- (16) Xylol—two changes—1 minute each.
- (17) Mount in balsam, gum damar, or clarite.

Note. To get best results, after fixation the slides or coverslips should be agitated frequently in each solution except while in the stain.

- (18) Tergitol-hematoxylin.
 - (a) Stock solution: 0.5 gm of hematoxylin crystals dissolved in 10 ml of 95 percent alcohol.
 - (b) Tergitol-hematoxylin staining solution: Dilute the stock solution 1 to 10 with distilled water, and ripen naturally by aging or immediately by the addition of a minute quantity of sodium bicarbonate. To every 30 to 40 ml of this solution, add 1 drop of tergitol #7 just before use. (Tergitol-hematoxylin can be used more than once, but due to the fact that it is unstable it should not be kept longer than 24 hours.)

453. Culture Methods

Culturing *E. histolytica* may be of assistance in establishing a diagnosis, but negative cultures alone cannot be accepted as indicating that the patient is free from infection. (Not frequently negative cultures result from overgrowth with bacteria or *Blas-tocystis*.) When *E. histolytica* does grow in culture its morphologic characteristics may become somewhat altered.

a. MEDIA.

- (1) *Boeck-Drbohlav medium*. This is the medium most generally used for culturing the intestinal protozoa of man. In it many amebae and intestinal flagellates, with the exception of *Giardia lamblia*, survive and multiply. Materials required to prepare it are as follows:

(a) *Reagents.*

1. Eggs.

2. *Sterile Ringer's solution.* This is prepared according to the following formula:

Sodium chloride	(NaCl)	8.0 gm
Potassium chloride	(KCl)	0.2 gm
Calcium chloride	(CaCl ₂)	0.2 gm
Magnesium chloride	(MgCl ₂)	0.1 gm
Monosodium phosphate	(NaH ₂ PO ₄)	0.1 gm*
Sodium bicarbonate	(NaHCO ₃)	0.4 gm*
Distilled water	(H ₂ O)	1,000 ml

The solution is autoclaved at 15-pound pressure for 20 minutes and allowed to cool.

3. *Modified sterile Ringer's solution.* This is prepared by adding 0.25 gm of Loeffler's dehydrated blood serum to 1,000 ml of Ringer's solution which should be made up in addition to the Ringer's solution of the preceding section. Boil the mixture of serum and buffer-free Ringer's solution for 1 hour to facilitate solution of the serum, filter, and autoclave for 20 minutes at 15-pound pressure; then add filtered buffer as before.

4. *Sterile Chinese rice flour.* The rice flour is sterilized by placing about 5 gm in a test tube and plugging it with cotton. The flour is distributed evenly and loosely over the inner surface of the tube by shaking, and then sterilized in a horizontal position in dry heat at about 90° C. for 12 hours, using intermittent sterilization—three periods of 4 hours each. The flour remains white if not overheated.

(b) *Preparation.* Wash four eggs thoroughly, rinse, and brush well with 80 percent alcohol. Break into a sterile Erlenmeyer flask containing sterile glass beads and 50 ml of sterile Ringer's solution. Emulsify completely by shaking the flask. Place about 4 ml of this material in each test tube and sterilize as follows (using autoclave as inspissator): Place the tubes in a preheated autoclave in such a position as to produce a slant of about 25 to 35 millimeters, close the door, turn on the steam, and open the outside exhaust valve. At the first appearance of steam from valve, close it, and allow the pressure to rise to 15 pounds; then shut off the steam and allow the pressure to decline to zero; re-

* These ingredients are dissolved in sterile distilled water, filtered through a Seitz or Berkfeld filter and added subsequently.

move the tubes from the autoclave. Repeat on 3 successive days, storing the tubes at room temperature between sterilizations.

To each solid slant add enough modified Ringer's solution (about 5 or 6 ml) to cover the slant completely. Incubate at 37° C. for 24 hours to determine sterility before adding the sterile Chinese rice flour. The flour is added by taking up 0.25 ml into a clean, sterile, dry, wide-bore 1-ml pipette and discharging it into the liquid medium by tapping the pipette against the inside wall of the tube. The tubes are again incubated at 37° C. for 24 hours to test for sterility.

(2) *Cleveland and Sanders medium, modified.*

(a) *Reagents.*

1. Entamoeba medium, dehydrated.

2. *Serum-saline solution.* This is a M/30 phosphate-salt solution (pH 8) prepared by dissolving 11.23 gm $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.26 gm KH_2PO_4 and 8 gm NaCl in distilled water to make 1 liter. Autoclave at 15 pounds for 20 minutes, cool and add 10 parts to 1 part of sterile (horse) serum.

3. Sterile rice flour, prepared as in paragraph 453.

(b) *Preparation.* Dissolve 33 gm dehydrated medium in 1 liter of distilled water, and dispense in tubes in a quantity which gives a slant of medium length with no butt. Autoclave and slant, leaving at room temperature several days to harden. Add enough serum-saline solution to cover three-fourths of slant and add 2 or 3 loopfuls of rice flour to each tube. Incubate at 37° C. to test sterility, and then store in refrigerator.

b. **CULTURING AND TRANSFERRING.** Inoculate the liquid portion of the medium with a portion of the stool about the size of a pea. Incubate at 37° C. and examine at the end of 24 and 48 hours. If subsequent cultures are to be maintained, transfer every 48 hours. Approximately 0.5 ml of the fluid medium taken from the bottom of the tube should be used for each transplant. Amebic cultures that are not positive at the end of 48 hours should be transferred and further examined as follows: Allow the culture to remain in the incubator for 2 hours, then without unduly disturbing it, remove all but 0.5 ml of the supernatant fluid by means of a sterile pipette equipped with a rubber nipple. Wash the slant with the remaining fluid and then transfer it to a new medium. The resulting culture should then be examined at 24 and 48 hours before calling it "negative."

454. Summary of Methods Used In Diagnosis of Amebae

a. FRESH, UNSTAINED, WET MOUNT. The fresh, unstained, wet mount prepared as outlined under general methods should be examined systematically, covering the slide without repeating any microscopic field. Amebae in the live state have a particular refractive, translucent, granular appearance that easily differentiates them from other objects in the field. The trophozoites and cysts of the amebae may be detected with the low-power objective, and then a switch made to the high-power objective to determine the detailed characteristics. Bar-shaped chromatoid bodies are diagnostic of unstained *E. histolytica* cysts. The features outlined below will be found to be of great aid in species determination:

- (1) Size and color.
- (2) Granular nature of cytoplasm and presence of cell inclusions.
- (3) Nuclei—number and visibility.
- (4) Type of motility—active, sluggish, progressive, or non-progressive.
- (5) Pseudopodia—single, multiple, clear, or granular.
- (6) Formation of pseudopodia—slow or explosive.
- (7) Presence or absence of red blood cells or bacteria in cytoplasm.
- (8) Presence and characteristics of chromatoid bodies inside the cysts.

b. FRESH, IODINE-STAINED, WET MOUNTS. Since iodine distorts trophozoites, the usefulness of this preparation is limited to the study of the cysts. It enables the observer to count the nuclei (8 in mature *E. coli* and 4 in *E. histolytica*) and to determine the placement of the intranuclear karyosome (eccentric in *E. coli* and central *E. histolytica*). The glycogen in the young cysts is stained light to dark brown. Chromatoid bodies do not stain with iodine; in fact, they often become less apparent than when unstained.

c. SMEARS STAINED WITH IRON-HEMATOXYLIN. See paragraph 452.

d. CULTURE. There are a number of cultural methods that may be helpful in establishing a diagnosis of *E. histolytica*. (See par. 453.) Usually, however, it is preferable to make direct microscopic examinations on several stools or on smears obtained with the aid of the sigmoidoscope.

e. CONCENTRATION OF CYSTS. This procedure should be applied preferably to formed or semiformed stools.

f. COMPLEMENT-FIXATION. The complement-fixation test for *E. histolytica* infection is still in the experimental stage, and few

laboratories are equipped for it. In the hands of experts, however, it may be of some value.

g. **CHARCOT-LEYDEN CRYSTALS.** Charcot-Leyden crystals in the stools are indicative of chronic inflammation, and often occur in amebiasis.

h. **DIFFERENTIATION OF AMEBIC AND BACILLARY DYSENTERY.** This can presumptively be done by observing the general character of the fecal exudate. However, one disease may complicate the other, and amebic dysentery in the presence of severe bacterial secondary invasion of the ulcers may have many of the characteristics of bacillary dysentery. Therefore, every effort should be exerted to demonstrate the causative organism in each case. The main differential points are shown in table LVI.

Table LVI. Differential Diagnosis of Fecal Elements in Bacillary and Amebic Dysentery¹

Element	Bacillary dysentery	Amebic dysentery
Blood.....	Varying amounts.....	Small amounts to actual hemorrhage.
Polymorphonuclear neutrophils.	About 90 percent of exudate. Many show nuclear degeneration (ringing). Cytoplasm frequently contains fat.	Few. Cytoplasm of some of those present shows degenerative changes and in such the nuclei may appear pyknotic.
Endothelial macrophages.	Present in varying numbers. Actively phagocytic, frequently contain erythrocytes and leucocytes. Undergo toxic degeneration—"ghost cells."	Not seen except in cases also having bacterial dysentery.
Plasma cells.....	Present; relatively more abundant early.	Present in small numbers.
Pyknotic bodies.....	Proportionately insignificant, but are found.	Constitute about 80 percent of cellular elements.
<i>E. histolytica</i> trophozoites.	Absent unless the two diseases are both present.	Present and must be found to make diagnosis.
Amount of exudate actual hemorrhage excluded.	Small to massive exudate, may be a large part of the stool.	Small.
Bacterial content.....	Low—due to bacterial flushing.	Very high, usually.

¹ Callender, G. R., *The Cytological Diagnosis of Dysenteric Conditions and Its Application in the Military Service*. The Military Surgeon, June, 1925.

455. Confusing Objects

a. Tissue cells derived from the host, or ingested as food, may at first glance appear as amebae in the stools. Macrophages may be found containing phagocytized red blood cells, but a critical examination will reveal their typical nuclear structure and the absence of ameboid movement. Epithelial cells are pale in color and have nuclear characteristics that easily differentiate them.

b. Vegetable elements such as starch grains, pollen grains, yeast cells, etc., have a certain definiteness of outline and structure that should lead to no confusion. However, certain yeast cells, such as *Blastocystis hominis*, may be confused with cysts of amebae. The occurrence of budding and the peculiar arrangement of their nuclei should aid in differentiating them.

456. Direction for Submitting Specimens of Feces by Mail To Central Laboratory

a. ROUTINE EXAMINATION FOR INTESTINAL PARASITES. Submit stool specimens in a 60 ml wide-mouth screw-top bottle and do not fill the bottle more than half full. Oily specimens are unsatisfactory. Mail in a double mailing case with packing cotton to protect against breakage and leakage.

b. FECAL SMEARS FOR HEMATOXYLIN STAINING. In addition to the above specimen, fixed smears of liquid or semiliquid stools should also be submitted to enable an examination for the trophozoite forms of protozoa. Make *thin* fecal smears on two slides with a small brush or applicator, and *while still wet*, immerse in warm Schaudinn's solution. (See par. 352.) Remove after 15 minutes and, *without allowing to dry*, place back to back and transfer to a suitable bottle completely filled with 70 percent ethyl alcohol and enough cotton to prevent the slides from being jostled about; then mail to the central laboratory for staining and examination.

Section III. FLAGELLATES OF BLOOD AND OTHER TISSUES

457. General

The flagellates found in the blood and other tissues of man belong to the genus *Leishmania* and the genus *Trypanosoma*. These organisms undergo changes in form during certain stages of their life cycles. Note that the genus *Trypanosoma* may assume any of the forms and that the genus *Leishmania* has only two forms (see fig. 31).

GENERIC TITLES	FLAGELLATE	TYPES
TRYPANOSOMA HERPETOMONAS CRITHIDIA LEISHMANIA LEPTOMONAS	 TRYPANOSOME	IN MAN
	 TRYPANOSOME	IN INSECT HOST
	 CRITHIDIA	
	 LEPTOMONAS	
	 LEISHMANIA	
	 LEISHMANIA	IN MAN

Figure 31. Classification of Trypanosomes and Leishmanias (modified after Wenyon).

458. Leishmanias

There are three generally recognized species of *Leishmania* infecting man which, although causing distinct diseases, appear identical under the microscope. The diseases produced in man, however, are clear-cut, there being three types of lesions: visceral, cutaneous, and mucocutaneous.

a. *LEISHMANIA DONOVANI* (VISCERAL FORM). Infection with this parasite is called "kala-azar" and is fairly common in the countries bordering the Mediterranean and in India, northern China, and southern Russian Turkestan; it has also been reported from South America. The Mediterranean type chiefly affects children but many adults harbor the parasite and it is not uncommon in aged people. The disease is chronic and is characterized by anemia, loss of weight, and marked enlargement of the spleen. The parasites are found in the reticuloendothelial cells of the liver, spleen, and lymph nodes and occasionally in the white cells circulating in the blood. It appears probable that flies of the genus *Phlebotomus* transmit the disease to man through their bites. (See par. 495.)

- (1) *Morphology*. In the tissues this species is a very small (2 to 3 μ) round or oval body, with a sharp outline and poorly staining cytoplasm. With Wright's stain, the cytoplasm stains a pale blue, the nucleus appearing as several bright red granules. The parabasal body stains deep purple and is the only other definite structure clearly visible in the parasite in man. (See fig. 32.)

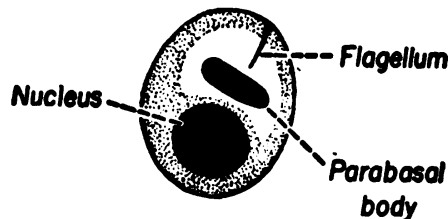


Figure 32. Diagram of *Leishmania* cell.

- (2) *Diagnostic methods*. The laboratory methods of diagnosis are, in order of reliability, as follows: culture of material obtained from spleen, liver, or sternal bone marrow, examination of stained material obtained from the above organs, the aldehyde test, culture of peripheral blood, and examination of stained blood films. Complement-fixation and precipitin tests have been used by certain workers with some degree of success. In consideration of the patient's safety and technical simplicity, the tests should be performed as follows:
 - (a) *Blood*. *Leishmania* are rarely recovered in the circulating blood. When present they are usually found in mononuclear and polymorphonuclear leucocytes. Attempts to cultivate the parasites should be made by inoculating tubes of NNN medium (*d below*) with blood concentrated by medium-speed centrifugation (800 rpm).

- (b) *Aldehyde test.* To 1 ml of clear serum obtained from the blood of a suspected case add 2 drops of formalin (40-percent formaldehyde solution) and shake. In advanced cases of kala-azar, the serum will become opaque and jell in 3 to 30 minutes, but early in the course of the infection it may become opaque or milky without jelling. Although this test may also be positive in malaria, tuberculosis, and leprosy, it is nevertheless of value.
- (c) *Sternal, hepatic, and splenic puncture.* These are procedures that should be done by a competent medical officer. Sternal puncture is safest and almost as reliable as splenic puncture. Hepatic and particularly splenic punctures are dangerous owing to the possibility of causing uncontrollable hemorrhage. The material obtained is spread on a slide in as thin a layer as possible, stained with Wright's or Giemsa's stain, and examined under a microscope with the oil immersion lens. The material may also be used to inoculate slants of NNN medium. (See (d) below.)
- (d) *Culture.* The NNN (triple N) medium is prepared as follows:

Agar	14 gm
Sodium chloride	6 gm
Distilled water	900 ml

Mix and dissolve by means of heat, then tube in 6 ml amounts and autoclave for 30 minutes under 15 pounds of steam pressure. Remove the tubes, and cool to 48° C. Then, under aseptic conditions, add 2 ml of sterile defibrinated rabbit's blood to each tube, mix well, and slant. Slanted tubes should be placed in the ice box to cool and harden so they will have the maximum amount of water of condensation. When cool, the cotton plugs of each tube should be covered with a rubber cap, or paraffin to prevent evaporation of the water. Inasmuch as trypanosomes will not grow in the presence of bacterial contamination, the tubes should be tested for sterility by incubation for 24 hours before they are used.

The material suspected of containing *Leishmania* is planted at the bottom of the slants in the water of condensation and incubated at 22° to 25° C. for 3 to 14 days.

b. **LEISHMANIA TROPICA (CUTANEOUS FORM).** Infection with this parasite, which is common in the Mediterranean area and in

Africa, is known as oriental sore, Delhi boil, and bubo. Although the organisms may occasionally be found in superficial scrapings from the base or margins of the ulcer, they can be found, with any degree of regularity, only in cells taken by puncture of the indurated margins of the ulcer. Material so obtained is spread on a slide and stained and examined as for *L. donovani*. Since the organisms are very scarce, much patience must be used in the search. The parasite may be cultivated in the NNN medium, but usually too many bacteria are present. The infection may be transmitted by contact, mechanically by a number of flies, and biologically by the sandfly (*Phlebotomus*).

c. *LEISHMANIA BRASILENSIS* (MUCOCUTANEOUS FORM). This organism produces mucocutaneous leishmaniasis or espundia of Central and South America. Its laboratory diagnosis is the same as that for *L. tropica*: examination of stained material and cultivation on NNN medium. The method of transmission is apparently the same as that of *L. tropica*.

459. Trypanosomes

Trypanosomes are flagellate protozoa of a somewhat elongate shape, tapering at both ends. Along the margin is an undulating membrane, to which is attached the flagellum. The flagellum

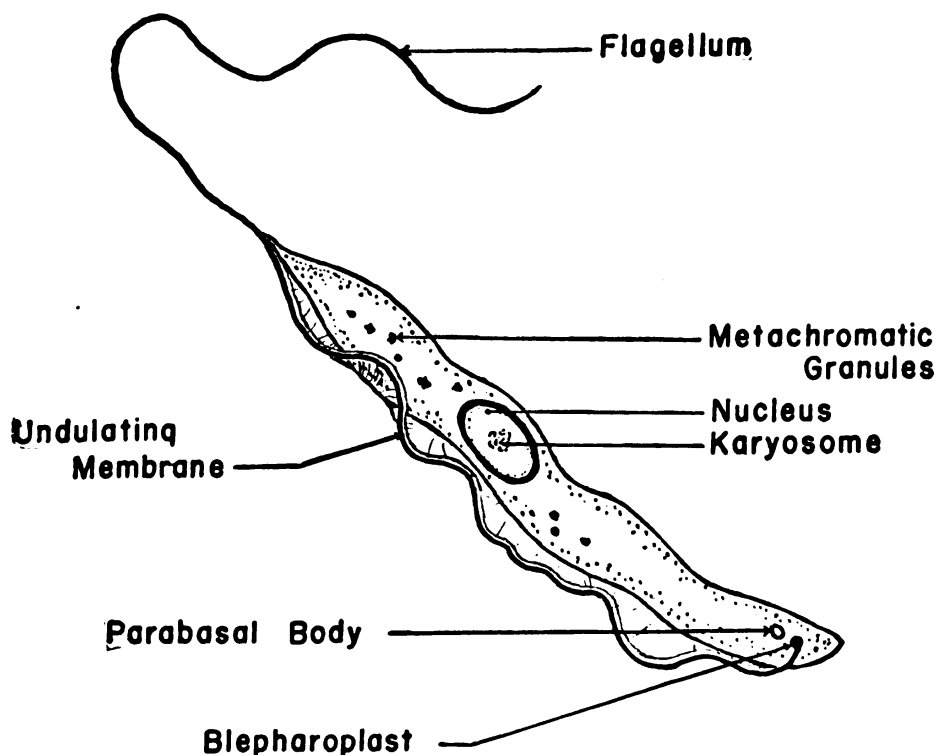


Figure 33. Diagram of a trypanosome.

arises from a small granule, the blepharoplast, near the posterior end of the organism. It then passes along the free margin of the undulating membrane and may emerge at the anterior end of the organism as a free flagellum of variable length. Just posterior to the blepharoplast there is usually a dark, shining, round or rodlike body known as the parabasal body. The nucleus of the organism is round or oval and centrally located. The cytoplasm is clear but may contain granules that stain red with Giemsa's stain. Figure 33 shows the internal structure of a trypanosome.

The trypanosomes of medical interest are *Trypanosoma gambiense* and *T. rhodesiense*, the causative organisms of African sleeping sickness, and *T. cruzi*, the causative organism of Chagas disease. (See table LVII).

a. **TRYPANOSOMA GAMBIENSE AND T. RHODESIENSE (AFRICAN SLEEPING SICKNESS).** The diseases produced by these two trypanosomes have several distinguishing characteristics, but for the sake of simplicity they will be considered together as African sleeping sickness.

- (1) *Life cycle.* The location of the trypanosomes in man depends on the stage of the disease. During the febrile or "glandular" stage they are located in the blood and lymph; later, when the cerebral or sleeping sickness stage is reached, they are to be found only in the cerebrospinal fluid. The organisms, which vary in length from 15 to 33 microns, multiply by longitudinal division while in the body fluids. The vectors, species of tsetse flies (*Glossina*), become infected by ingesting trypanosomes with their blood meals. The organisms multiply in the midgut of the fly, migrate up the digestive tract and enter the salivary glands, where they later transform into metacyclic forms, which are infective for man. This cyclical development in the insect vector requires about 3 weeks.
- (2) *Diagnostic methods.* The diagnostic method to be employed is dependent on the stage of the disease in the patient. Inasmuch as successful treatment during the sleeping-sickness stage is difficult, if not impossible, it is imperative to make a correct diagnosis early in the course of the disease.
 - (a) *Febrile stage.*
 1. *Blood and lymph.* The direct examination of blood and lymph-node fluid reveals most of the infections during this stage. Owing to the motility of trypanosomes, the organisms are frequently more easily

Table LVII. *Trypanosomes of Medical Importance*

Species	Length	Definitive host	Intermediate host	Geographical distribution	Susceptible animals	Culture	Disease
<i>T. gambiense</i>	15 to 30 μ	Man and domestic animals.	Tsetse fly, <i>Glossina palpalis</i> .	Tropical Africa but mostly western and central.	All laboratory animals except monkey.	Poor on NNN	Gambian sleeping sickness.
<i>T. rhodesiense</i>	12 to 35 μ	Man, big game animals and antelope.	Tsetse fly, <i>G. morsitans</i> .	Eastern Africa, eastern Rhodesia, etc.	All laboratory animals except monkey.	Poor on NNN	Rhodesian sleeping sickness.
<i>T. cruzi</i>	20 μ	Man, cat, dog, armadillo, opossum, wood rats and bats, monkeys and numerous others.	Kissing bug, (<i>Panstrongylus megistus</i>).	South and Central America (reservoir hosts in United States).	Guinea pigs, white rats, and monkeys.	Good on NNN	Chagas' disease.

observed in fresh unstained fluid than in stained preparations. Thick and thin films may be prepared and stained as for malaria parasites. (See pars. 463 and 464.) In obtaining lymph-node fluid an enlarged node is punctured aseptically with a Luer syringe equipped with a 19-gauge needle and some of the juice aspirated from it. Trypanosomes in the blood may be concentrated by centrifugation. Collect 10 ml of venous blood and centrifuge at 900 to 1,000 rpm for 3 minutes. The supernatant is pipetted off and centrifuged a final time at 1,800 to 2,000 rpm for 20 minutes. The sediment is examined either stained or unstained. If cultivation of the sediment is to be attempted, aseptic precautions should be followed.

2. *Animal inoculation.* White rats, the blood of which is free from trypanosomes (*T. lewisi*) or guinea pigs, may be inoculated intraperitoneally with 1 to 5 ml of the patient's blood, the size of the inoculum depending on the size of the animals used. The inoculation period in the laboratory animal depends on several factors, for example, the size of the inoculum and the species of trypanosome. It may be necessary to examine the inoculated animal's blood on several occasions over a period of a month or more. *T. rhodesiense* is more virulent than *T. gambiense* for laboratory animals, and exhibits a larger percentage of posteriorly placed nuclei when found in the blood of the rat.
3. *Culture.* Blood and lymph-node juice can be inoculated into tubes of NNN medium, but the cultivation of *T. gambiense* and *T. rhodesiense* is difficult.
- (b) *Sleeping-sickness stage. Cerebrospinal fluid.* Five milliliters of fluid obtained by lumbar or cisternal puncture is centrifuged at 1,500 to 2,000 rpm for 20 minutes. The sediment is examined fresh or stained, and may be inoculated intraperitoneally into suitable animals. An increase in the number of leucocytes in the cerebrospinal fluid is suggestive but not diagnostic.

b. **TRYPANOSOMA CRUZI (CHAGAS' DISEASE.)** This is a trypanosome infecting man in tropical and subtropical South and Central America. Although this is primarily a childhood disease, it is sometimes found in adults.

(1) *Life cycle.*

- (a) In man, *T. cruzi* as seen in the peripheral blood is a trypanosome about 20 microns in length. In blood films, its body tends to be shaped like the letter "C" and the posterior end is sharply pointed. The parabasal body is oval in shape and very conspicuous. There is a free flagellum. The nucleus is oval and centrally located. There may be some variations in the width of the body, since the young forms are narrower than the old. After a variable period in the blood stream, the mature trypanosomes invade the muscle fibers or other tissue cells, assume the leishmanial form and multiply by splitting lengthwise. This multiplication of the leishmanial stage continues until the tissue cells become distended with parasites and rupture. Each parasite then develops a short flagellum, the body lengthens, the parabasal body moves posteriorly, and the young trypanosome returns to the blood stream or invades other tissue cells. There is considerable local tissue damage and destruction due to the invasion of tissue cells by this parasite.
 - (b) In triatomid insects a complicated development cycle takes place that results in the appearance in the hind-gut of "metacyclic" trypanosomes, forms which are infective for man. When the insect feeds again, it deposits a small drop of fluid from the rectum upon the skin of the human being. This fluid excrement may contain great numbers of "metacyclic" trypanosomes, which are capable of passing through intact mucous membranes or small skin abrasions into the blood stream, thus infecting the individual. Persons may inoculate themselves by accidentally rubbing the deposited feces into the puncture wound at the feeding site or into the eye.
- (2) *Diagnostic methods.* The diagnosis of Chagas' disease by the demonstration of *T. cruzi* is essentially the same as that for African sleeping sickness but is far more difficult. Trypanosomes are in the peripheral blood only during the acute stage of the disease and during febrile periods of the chronic condition. If direct examination of the blood is negative, 0.05 to 2 ml of the patient's blood should be inoculated into guinea pigs or into white rats free from *T. lewisi*. In positive cases the organisms will be found in the blood of these animals in from 1 to 4 weeks. The lymph nodes are less often involved in this

disease and need not be examined. If nervous symptoms happen to be present, examination of the cerebrospinal fluid may reveal the organisms. The cultivation of *T. cruzi* on NNN medium is more successful than that of *T. gambiense* and *T. rhodesiense*. If the antigens are available complement-fixation tests can be performed (Kelser: Am. J. Trop. Med. 16:405, 1936).

Section IV. MALARIAL PARASITES

460. General

a. A positive diagnosis of malaria in man should always be made by the demonstration of malarial parasites in stained blood films. Since there are three important kinds of malarial parasites, and since all these may appear in the blood in more than one form, it is important to learn the diagnostic features of each kind in all its common stages. The following glossary will be helpful if consulted during the reading of subsequent paragraphs in this section.

b. GLOSSARY OF TERMS USED IN MALARIOLOGY:

Anopheline. The particular kind of mosquitoes that transmit malaria from man to man.

Appliqué or accolé form. A young ring of *Plasmodium falciparum* found at the periphery of a red cell.

Band form. The bandlike position of the malarial parasite across the red cell seen in *P. malariae* infections.

Black-water fever. A highly fatal disease associated with *P. falciparum* infections in which hemoglobin is lost rapidly through the urine.

Chromatin. Nuclear material, which in malarial parasites, is sometimes scattered, rather than in a distinct compact nucleus. With Wright's and Giemsa's stains, plasmodial chromatin stains red.

Crescents. Gametocytes of *P. falciparum*, recognized by their typical shape.

Exoerythrocytic stage. Applies to early developmental stages believed to occur outside the red cells, especially in cells of the reticuloendothelial system.

Gametocyte. A sexual form of the malaria parasite in man. Fertilization occurs only in the stomach of anopheline mosquitoes.

Macrogametocyte. A female malaria parasite.

Malaria. A group of diseases with chills and fever caused by several kinds of blood parasites called plasmodia.

Malarial pigment. Brownish to blackish granules deposited in the cytoplasm of malarial parasites during their growth.

Maurer's dots. Spare red-staining coarse granules occasionally seen in the cytoplasm of red cells infected by *P. falciparum*.

Merozoite. One of the subdivisions of a schizont. When the red cell ruptures, each merozoite enters a new red cell and forms a young ring form of the next generation.

Microgametocyte. A male malarial parasite.

Multiple infection. The presence of more than one malarial parasite in a single red cell.

Plasmodium. The name of the genus of *Protozoa* to which malarial parasites belong.

***P. falciparum*.** The parasite causing estivoautumnal, malignant tertian, subtertian, or pernicious malaria. Chills and fever recur at 48-hour intervals. *This is by far the most dangerous form of malaria.*

***P. malariae*.** The parasite causing quartan malaria. Chills and fever recur at 72-hour intervals.

***P. ovale*.** The parasite causing a rare benign form of tertian malaria, found chiefly in Africa.

***P. vivax*.** The parasite causing benign tertian malaria. Chills and fever recur at 48-hour intervals.

Presegmenters. Schizonts at an early stage of division having divided chromatin but intact cytoplasm.

Ring or ring stages. Early forms of malarial parasites in the red cells. Each parasite's circular mass of cytoplasm with its chromatin dot at one side resembles a signet ring.

Schizogony. A sexual division of mature trophozoites into merozoites.

Schizont. A malarial parasite undergoing asexual division, characterized by dividing masses of chromatin.

Schuffner's granules. Abundant pink-staining granules often seen in the cytoplasm of red cells infected by *P. vivax* or *P. ovale*.

Segmenters. Mature schizonts having divided chromatin and cytoplasm.

They consist of merozoites and clumped pigment.

Sporozoites. Spindle-shaped malarial parasites injected into man by bites of infected anopheline mosquitoes.

Trophozoites. Growing stages of malarial parasites in the red cells.

461. Life Cycle of Malarial Parasites

a. In order to understand the diversity of forms of malarial parasites found in a blood film, it is necessary to know something about the life cycle of malarial parasites. (See fig. 34.)

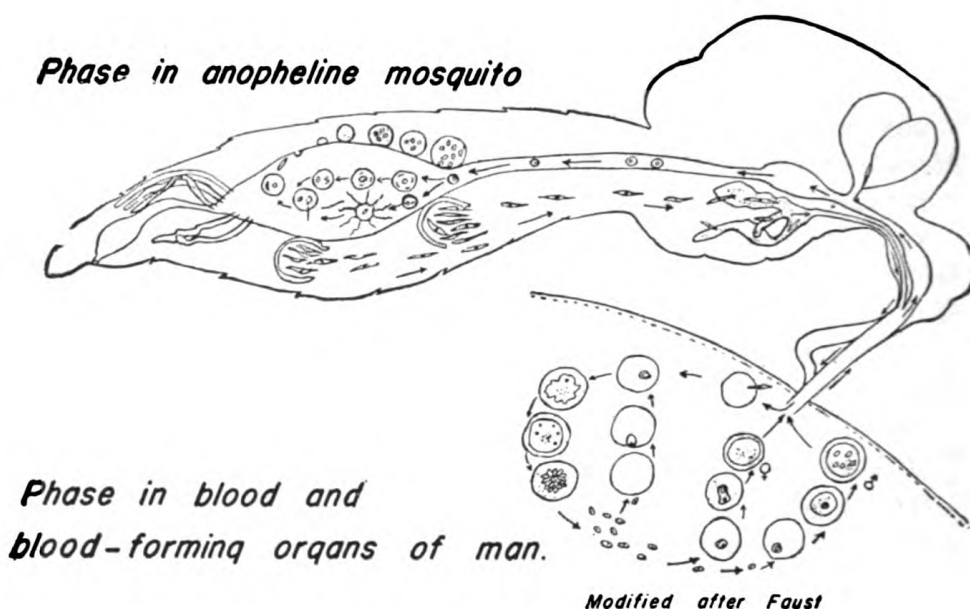


Figure 34. Life cycle of malaria parasites.

When the infected female anopheline mosquito bites man, she injects malarial sporozoites into the wound. These enter the blood stream and a number of days later give rise to the first generation of ring forms in the corpuscles. Each ring form (trophozoite stage) grows until at the end of 48 or 72 hours it fills much of the red cell, when the chromatin divides and the parasite becomes a schizont. This schizont continues to divide into many small segments called merozoites. The red cell finally ruptures, liberating the merozoites, each of which enters a new red cell to repeat the asexual cycle.

b. Later in the disease some of the merozoites give rise to male gametocytes (microgametocytes) and female gametocytes (macrogametocytes) which circulate without further development until they are taken up by another mosquito or die. Within the mosquito they mate and produce thousands of sporozoites, thus completing the sexual cycle.

c. The forms of malarial parasites seen in blood films are therefore as follows: early rings; growing trophozoites; early schizonts (presegmenters); mature schizonts, late segmenters containing merozoites); male gametocytes; and female gametocytes.

- (1) In all these forms the cytoplasm stains blue and the chromatin red with Wright's and Giemsa's stains.
- (2) Sporozoites are never seen in blood films.
- (3) Blood platelets, small lymphocytes, and debris are sometimes confused with malarial parasites by inexperienced

workers. Careful attention to the minute structural details of true Plasmodia should eliminate such errors.

- (4) All six of the above-listed forms may appear simultaneously in a blood film from patients with *P. vivax* or *P. malariae* infection. But with *P. falciparum* infection one usually sees only rings or crescents (gametocytes), or both, the growing trophozoites and schizonts being confined in the internal organs. To detect the latter it is sometimes necessary to perform splenic or sternal punctures.
- (5) Female gametocytes have compact chromatin and closely packed malarial pigment, whereas in males both these elements are more diffusely scattered. Gametocytes of *P. vivax* and *P. malariae* are round, in contrast to the crescents of *P. falciparum*.
- (6) Additional diagnostic features of the various species of *Plasmodium* are summarized in tables LVIII and LIX. The colored plates (figs. 36, 37, and 38) should also be frequently consulted.

Table LVIII. Summary of the Diagnostic Characteristics of Malarial Parasites when Seen in Stained Blood Films

Characteristic	<i>P. vivax</i>	<i>P. ovale</i>	<i>P. malariae</i>	<i>P. falciparum</i>
Enlarged red cells.....	+	+	—	—
Fringed or oval red cells ²	—	+	—	—
Schuffner's granules.....	+	+	—	—
Large irregular rings.....	+	—	—	—
All forms in peripheral blood.....	+	+	+	—
Band forms.....	—	+	+	—
Maurer's dots.....	—	—	—	+
Double chromatin dots.....	—	—	—	+
Appliqué forms.....	—	—	—	+
Multiple infection.....	Rare	—	—	+
Oval presegmenters.....	—	+	—	—
Crescents.....	—	—	—	+
Divisions of chromatin.....	12-24 (16) ¹	6-12 (8) ¹	6-12 (8-10) ¹	6-30 (16) ¹

¹ Average.

² Fringed or oval red cells are less frequently seen in *P. vivax* infections but they are not rare.

Table LIX. Diagnostic Characteristics of the Three Principal Malaria Parasites of Man

P. vivax:

Infected red cells enlarged and pale (except youngest ring forms).

Trophozoites bizarre shaped and ameboid.

Schuffner's granules in cytoplasm of infected cells highly suggestive.

Table LIX — Continued

Merozoites number 12–24, average 16.
Pigment yellowish brown, and finely granular.
Gametocytes nearly or completely fill cell.
Rings relatively large and coarse.
Multiple infection of cells uncommon.

P. malariae:

Occasional or frequent band formation.
Malarial pigment abundant, very dark and coarsely granular.
Red cells not enlarged or pale.
Merozoites number 6–12 (rosette formation), average 8–10.
No Schuffner's granules or Maurer's dots.

P. falciparum:

Early rings very small, many with double chromatin dots.
Multiple infections characteristic.
Appliqué forms often seen.
Maurer's dots occasionally present.
Crescent-shaped gametocytes.
No enlargement or paling of red cells.
Intermediate forms rarely found in peripheral blood.
Merozoites number 6–30, average 16.

462. Laboratory Diagnosis of Malaria

a. The most favorable time to find malarial parasites in the blood in a clinical case of malaria is the period just before or at the beginning of the paroxysm. Most examinations are made on stained blood smears, but in an emergency, or if staining facilities are not available, the parasites may be seen in fresh unstained blood.

b. Suspected positive findings should be confirmed by the laboratory officer before being reported.

c. Quinine or other antimalarial drugs used in treatment within 4 days before taking the sample make it very difficult to demonstrate the parasites even by the thick-film method.

d. Repeat the examination as many times as necessary at 12-hour intervals to prove or disprove the diagnosis.

e. Use only glass slides that are chemically clean and free of scratches, grease or fogging.

463. Preparation of Blood Films

a. Wipe the finger with gauze moistened with 70 percent ethyl alcohol and allow the alcohol to evaporate completely. Puncture the skin, preferably using a No. 11 sterile operating knife blade, and wipe off the first drop of blood. To make a thin smear, touch the slide to the top of the drop, being careful that the slide does not touch the finger, and with another clean slide draw the blood

from the edge to the center of the slide. The thick film should be made simultaneously. A large drop of blood is collected on the opposite end of the slide, and this is stirred immediately with the corner of another slide until the blood is evenly spread over an area about 1 cm in diameter. (If thick films while still wet are the correct thickness, ordinary print should be visible through them.) A correctly prepared slide is shown in figure 35.

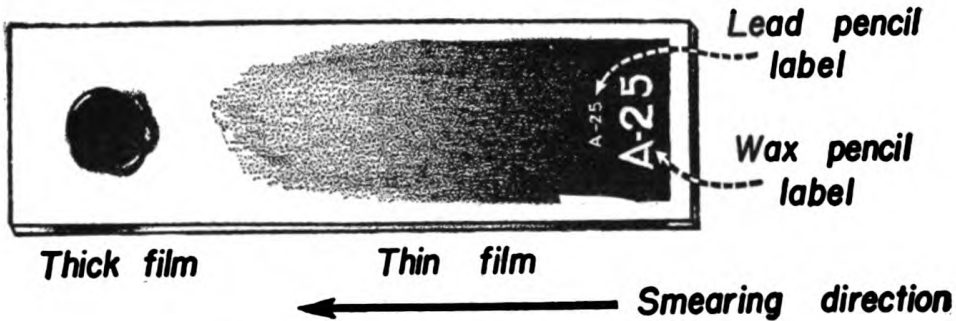


Figure 35. A correctly prepared blood film.

b. The name of the patient should be written with pencil on the thicker portion of the thin film. The slide should be placed flat in a slide box standing in a vertical position or covered with a Petri dish to prevent dust, debris, roaches, and flies from reaching the smears. The slide should be allowed to air dry for several hours. In case of emergency, however, the slide may be stained as soon as it is thoroughly dry, if care is taken in removing the slide from the stain and in washing. In this case the drying process may be hastened by placing the slide in an incubator.

464. Staining of Films

a. ROUTINE METHOD FOR THIN AND THICK FILMS.

- (1) The solution for staining is made up by adding 1 part of Giemsa stain to approximately 50 parts of buffered water (pH 7.0). This dilution will vary somewhat with each batch of stain. The staining solution should be made up fresh just before use. Fix the thin film with methyl alcohol before placing in the stain, *being careful not to allow the alcohol to spread to the thick film*. Allow to dry. and place the slide in the staining solution for 45 minutes. Remove, and dip the slide two or three times in buffered water (pH 7.0). Then place in a container with buffered water of a depth sufficient to *cover the thick smear only*. Allow to remain for 3 minutes. Remove slide and stand on paper towel or newspaper and allow to air dry. Do not blot.

(2) *Preparation of buffered water.*

- (a) Distilled water may be neutralized in a number of different ways. For this purpose two buffer solutions have been adopted—M/15 anhydrous disodium phosphate (Na_2HPO_4) and M/15 sodium acid phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$). These are made up as follows:

	<i>Gm per liter</i>
M/15 Na_2HPO_4	9.5
M/15 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	9.2

Note. Each may be kept indefinitely in a Pyrex glass stoppered bottle.

- (b) From these stock solutions the buffered water used in staining and rinsing the stained specimens is prepared. Always filter the buffers before adding the water to them. The buffered water is kept in well-stoppered glass bottles and made up fresh each week. The pH of the water is tested occasionally to check the accuracy of the buffer solutions.
- (c) The following proportions are used in buffering water at pH7.0: M/15 Na_2HPO_4 , 61.1 ml; M/15 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 38.9 ml, and distilled water, 900 ml.

(3) *Preparation of Giemsa stain.*

- (a) The ingredients are as follows:

Giemsa stain (dry powder)	0.6 gm
Methyl alcohol	50 ml
Glycerine	50 ml

- (b) Chemically clean and absolutely dry glassware must be used. First measure out 50 ml of glycerine in a graduated cylinder. Put a small portion of the dry powder in a mortar, add a small amount of glycerine and grind thoroughly. Pour off this portion into a sterile flask and continue the above process until all the stain has been ground with glycerine. It is wise to save a portion of the glycerine for rinsing the mortar and pestle in order to remove the last bit of stain. Place the glycerine-dye mixture in a water bath (55° – 60° C.) for at least 2 hours, preferably all day. The mixture may be shaken slightly from time to time while it is in the water bath.
- (c) Measure out 50 ml of methyl alcohol in a graduated cylinder. Use a portion of this to rinse the remains of the glycerine and dye from the mortar and pestle, pouring back this portion into the alcohol in the cylinder. This alcohol must be well stoppered until used.

(d) After removing the glycerine-dye mixture from the water bath, allow it to cool to room temperature and then add the methyl alcohol. Stopper tightly and age for from 10 days to 2 weeks. At the end of this period, filter the solution into chemically clean, absolutely dry bottles.

(e) Test for the proper dilution, label, and store for future use.

b. RAPID STAINING OF THICK FILMS. (Field Reference—J. W. Fields, Trans. Royal Soc. Trop. Med. and Hy. Vol. 35, No. 1, July, 1941). In this method, thick blood films are stained in such a manner that the stained parasites and leucocytes are contrasted against a background of laked hemoglobin. The differentiation of color is more clearly shown in the lower edge of the film toward which the hemoglobin has drained.

In anemic blood the chromatoid and reticular residues of immature red cells are stained and should not be confused with malarial parasites. Reduced hemoglobin content of the blood increases the staining time to as much as 10 seconds in cases of severe anemia.

(1) *Preparation of films.* The blood films should be about 2 cm in diameter, and not too thick. The films are ready to stain as soon as they are no longer obviously moist. Fixation is not necessary. Freshly prepared blood films stain better than those 1 or 2 days old.

(2) *Preparation of stains.*

Solution A:

Methylene blue	0.80 gm
Azur B (American) ¹	0.50 gm
Disodium phosphate (anhydrous)	5.00 gm
Potassium acid phosphate (anhydrous)	6.25 gm
Distilled water	500 ml

Solution B:

Eosin	1.00 gm
Disodium phosphate (anhydrous)	5.00 gm
Potassium acid phosphate (anhydrous)	6.25 gm
Distilled water	500 ml

¹ The German equivalent of American azur B is *Azure I*. Should azur B be unobtainable, it is possible to prepare a methylene blue-azur mixture of undefined composition from medicinal methylene blue. Solution A in that case may be prepared as follows:

Dissolve 1.3 gm of medicinal methylene blue and 5.0 gm of anhydrous disodium phosphate in 50 ml of distilled water.

Bring the solution to a boil and then evaporate over a water bath almost to dryness; add 6.25 gm of anhydrous potassium acid phosphate.

Add 500 ml of distilled water, stir until the dye is completely dissolved, and set aside for 24 hours.

Filter before use.

The phosphate salts are first dissolved in the distilled water, then the stain or stains are added. Solution of the azur B is aided by grinding in a mortar with a small quantity of the phosphate solution. The stains should be set aside for 24 hours, and after filtration they are ready for use. The same solutions may be used for many weeks without deterioration, but the eosin solution should be renewed when it becomes greenish from a slight carry-over of methylene blue.

(3) *Method of staining.*

- (a) Dip the film for 1 to 5 seconds into solution A.
- (b) Remove from solution A and immediately rinse by waving *gently* in clean water for a few seconds until stain ceases to flow from the film and the glass of the slide is free from stain.
- (c) Dip for 1 to 5 seconds into solution B.
- (d) Rinse by waving *gently* for 2 or 3 seconds in clean water.
- (e) Place vertically against a rack to drain and dry.

c. THE "JSB" METHOD OF STAINING BLOOD FILMS. The "JSB" (Jaswant Singh-Bhattacharji) method (modified) is a rapid technic which may be used for staining thick or thin films. The chief disadvantage of the method is that the stain fades in time.

(1) *Preparation of stains:*

(a) *Solution A:*

Medicinal methylene blue.....	0.5 gm
Potassium dichromate	0.5 gm
1 per cent H ₂ SO ₄	2.5 ml
(1 ml concentrated H ₂ SO ₄ and 99 ml water)	
Water	500.0 ml

Dissolve the methylene blue thoroughly in 500 ml water.

Add the 1 percent H₂SO₄, mix thoroughly and add the potassium dichromate. A heavy amorphous purple precipitate of methylene blue chromate forms. Heat in an autoclave at a temperature of 100° to 109° C. and at a pressure of 0 to 5 lbs. for 3 hours. If the color remains greenish, further heating for another hour or so is required. At the end of this period, the solution turns blue, which indicates almost complete polychroming. If the temperature is allowed to rise above 110° C., the oxidation of methylene blue may be carried too far and the solution will turn violet purple. When the solution has turned deep blue, allow

it to cool to room temperature. Then add 10 ml of 1 percent KOH or NaOH, drop by drop very gradually while constantly shaking the flask. After the total amount of the alkali has been added, transfer half the contents of the flask into another of the same capacity and continue to shake for 15 or more minutes. Pour from one flask to the other until the precipitate is dissolved and the solution has turned a deep blue with a violet irridescence. Leave at room temperature for 48 hours to mature and filter.

- (b) *Solution B*: Dissolve 1 gm of water soluble eosin in 500 ml tap water. Set aside for 48 hours and filter.

(2) *Directions for staining.*

(a) *Thin films.*

1. Fix smear in methyl alcohol for 1 minute and dry.
2. Place in solution A for 30 seconds.
3. Wash in water (pH 6.2 to 6.6).
4. Place in solution B for 1 second.
5. Wash in same water as in step 3.
6. Replace in solution A for 30 seconds.
7. Wash in same water as in step 3 until the smear has a pink background.

(b) *Thick films.*

1. Lake the thick film in water until it has a gray appearance, and dry.
2. Place in solution A for 10 seconds.
3. Wash in water (pH 6.2 to 6.6) for 2 seconds.
4. Place in solution B for 1 second.
5. Wash in same water as in step 3 for 5 seconds.
6. Wash in same water as in step 3 until the smear gives a pink background.

465. Examination of Films

a. COMBINED THICK AND THIN FILM. The following paragraphs are quoted from Wilcox.²

Of the two types of films, the *thin* film and the *thick* film, each has its place. It is much simpler for a beginner to distinguish the various forms on the thin smears where the characteristics of the red blood cells can be examined also. The thick film is a concentration method, the purpose of which is the diagnosis

²The *Malaria Parasites of Man*—mimeographed notes for class use, not published.

1. Normal sized red cell with marginal ring-form trophozoite.
2. Young signet-ring form trophozoite in a macrocyte.
3. Slight older ring-form trophozoite in red cell showing basophilic stippling.
4. Polychromatophilic red cell containing young tertian parasite with pseudopodia.
5. Ring-form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schuffner's stippling.
- 6, 7. Very tenous medium trophozoite forms.
8. Three ameboid trophozoites with fused cytoplasm.
- 9, 11, 12, 13. Older ameboid trophozoites in process of development.
10. Two ameboid trophozoites in one cell.
14. Mature trophozoite.
15. Mature trophozoite with chromatin apparently in process or division.
- 16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").
20. Mature schizont.
- 21, 22. Developing gametocytes.
23. Mature microgametocyte.
24. Mature Macrogametocyte.

Schuffner's stippling does not appear in all cells containing the growing and older forms of *P. vivax* as would be indicated by these pictures, but it can be found with any stage from the fairly young ring form onward.

Figure 36 — Development of malarial organism: Plasmodium Vivax.

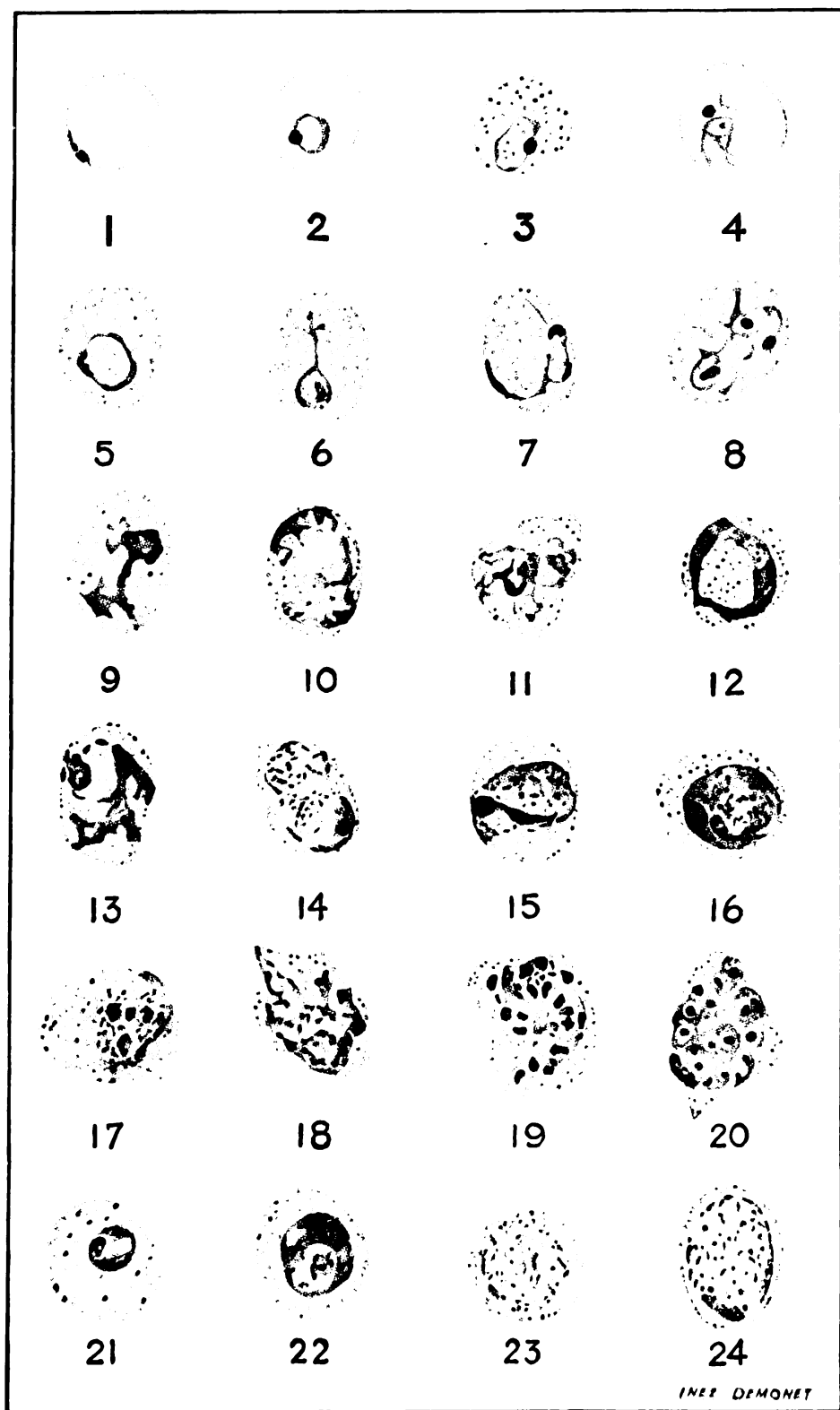


Figure 36. Development of malarial organism: *Plasmodium vivax*.

of malaria rather than the study of its parasites; so the microscopist must be familiar with the normal constituents of blood and with the morphology of the malarial parasites as demonstrated in the thin film, before attempting to examine thick films. One must take into consideration that the difference in the two techniques lies in the fact that the hemoglobin of the red cells is removed in the thick film staining process and that the outlines of red cells are thus obliterated. This leaves only a light blue or nearly colorless background against which clearly stand out the purple nuclei of the white cells, the purplish pink of the blood platelets and the characteristically stained parasites. There are two signals which attract the attention of the microscopist when examining either a thick or thin film for parasites. These are the purplish red or magenta color of the chromatin and the black or brown granules of the pigment. The blue of the cytoplasm, while not so obvious, is easily seen upon closer examination. After practice, the outlines of the red cells are not necessary as a guide, and one readily recognizes the characteristic shapes and colors of the parasites in thick films.

Frequently in a thick film the ring forms are not complete, i.e., only the chromatin dot with a portion of the cytoplasmic circle is visible. With searching, however, particularly around the edge of the smear, one usually finds forms that are typical and learns to identify the less characteristic forms. Sometimes, in slightly old or not very well stained specimens, one will find pigment alone or pigment associated in some instances only with cytoplasm, but such experiences are rare in clean preparations and are not evenly distributed throughout the smear as parasites may be. Artefacts, which may deceive the inexperienced, will be found frequently to lie above the blood plane. They may be refractile or may focus out of the field unevenly. According to Barber and Komp, a good general rule is not to consider anything a parasite which can be interpreted as an artefact.

In thick films at least a hundred microscopic fields are examined and the search is prolonged if anything suspicious of parasitism is seen. In thick films the suspicious things may be clumps of pigment, chromatin-like bodies, or an increased number of reticulocytes. These stain very nicely with Giemsa stain in the thick film technic and appear as a clear blue reticulum, though without a cell wall. In *thin films* one may suspect malaria if one sees clumps of pigment in the leucocytes, chromatin-like bodies in the red cells, increased polychromatophilia, basophilic stippling, central achromia of the red cells, variation in size and shape of the red cells, or nucleated red cells. In the differ-

1. Young ring-form trophozoite of quartan malaria.
- 2, 3, 4. Young trophozoite forms of the parasite showing gradual increase of chromatin and cytophasm.
5. Developing ring-form trophozoite showing pigment granule.
6. Early band-form trophozoite — elongated chromatin, some pigment apparent.
- 7, 8, 9, 10, 11, 12. Some forms that the developing trophozoite of quartan may take.
- 13, 14. Mature trophozoites—one a band form.
- 15, 16, 17, 18, 19. Phases in the development of the schizont ((presegmenting schizonts")).
20. Mature schizont.
21. Immature microgametocyte.
22. Immature macrogametocyte.
23. Mature microgametocyte.
24. Mature macrogametocyte.

Figure 37 — Development of malarial parasite: Plasmodium malariae.

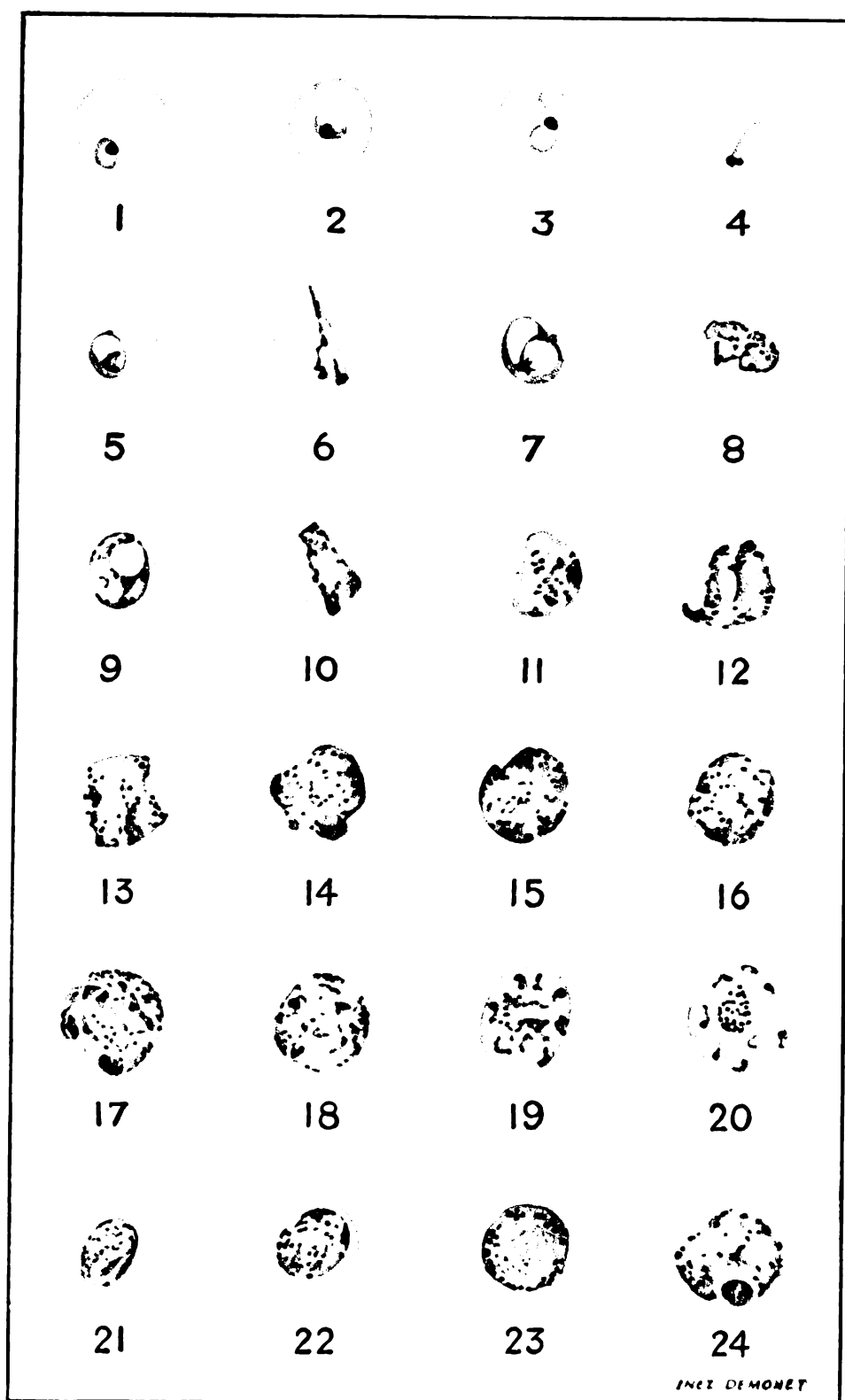


Figure 37. Development of malarial parasite: *Plasmodium malariae*.

ential count, the leucocytes, which are usually decreased in number, may present an increase in large monocytes and an immaturity of neutrophils. A leucocytosis does not exclude malaria, however, as the leucocyte count may rise during the febrile period or with some complicating factor, such as pneumonia.

The thick film has its advantages. It has been proved by experience that the thick film may increase by fifty percent the number of positives found in clinical cases. It is particularly valuable in finding the parasites of light infections or chronic cases. It concentrates a comparatively large amount of blood in a very small area, thus speeding examination. In indexes from endemic regions, one hundred to one hundred twenty-five microscopic fields per slide are examined and experience has shown that the first parasite is usually found within the first twenty to thirty fields. The experienced technician can examine one hundred fields on a good thick film in three or four minutes, whereas at least thirty minutes is spent on a thin film before calling it negative.

b. MICROSCOPIC EXAMINATION. To examine preparations made for malarial diagnosis, use the oil-immersion objective. Malarial parasites are best seen when the light coming through the substage is slightly reduced. The proper amount of light may be obtained by moving the slide until a blood platelet is centered in the field, then adjusting the substage so that the maximum definition of its morphological detail is obtained. Greater intensity of light may be preferred at times to distinguish colors more easily. In searching for the parasites, cover the slide in an orderly manner, moving back and forth over the smear so as not to repeat any field previously examined. *Never make a diagnosis on the first parasite found*; cover enough of the slide so that if two species of malarial parasites are present you will find them. If in doubt about any single abnormal parasite, remember that where there is one malarial parasite there are bound to be more, and careful search will usually reveal an easily recognizable form. When the technician has determined the presence of malarial parasites he should submit the slide to the laboratory officer for confirmation. No positive reports for malarial parasites should leave the laboratory except those slips signed by a responsible officer. If no officer is available and you are sure there are malarial parasites present, report as a suspected positive. Save the slide for final confirmation at a later date.

c. USE OF WRIGHT'S STAIN. Satisfactory slides may be obtained with this stain in the event that Giemsa's stain is not available.

1. Very young ring-form trophozoite.
2. Double infection of single cell with young trophozoites, one a marginal form, the other a signet-ring form.
- 3, 4. Young trophozoites showing double chromatin dots.
- 5, 6, 7. Developing trophozoite forms.
8. Three medium trophozoites in one cell.
9. Trophozoite showing pigment, in a cell containing Maurer's spots.
- 10, 11. Two trophozoites in each of two cells, showing variation of forms that parasites may assume.
12. Almost mature trophozoite showing haze of pigment throughout cytoplasm; Maurer's spots in the cell.
13. Aestivoautumnal "slender forms."
14. Mature trophozoite, showing clumped pigment.
15. Parasite in the process of initial chromatin diversion.
- 16, 17, 18, 19. Various phases in the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
- 21, 22, 23, 24. Successive forms in the development of the gametocyte usually not found in the peripheral circulation.
25. Immature macrogametocyte.
26. Mature macrogametocyte.
27. Immature microgametocyte.
28. Mature microgametocyte.

Figure 38 — Development of malarial parasite: Plasmodium falciparum.

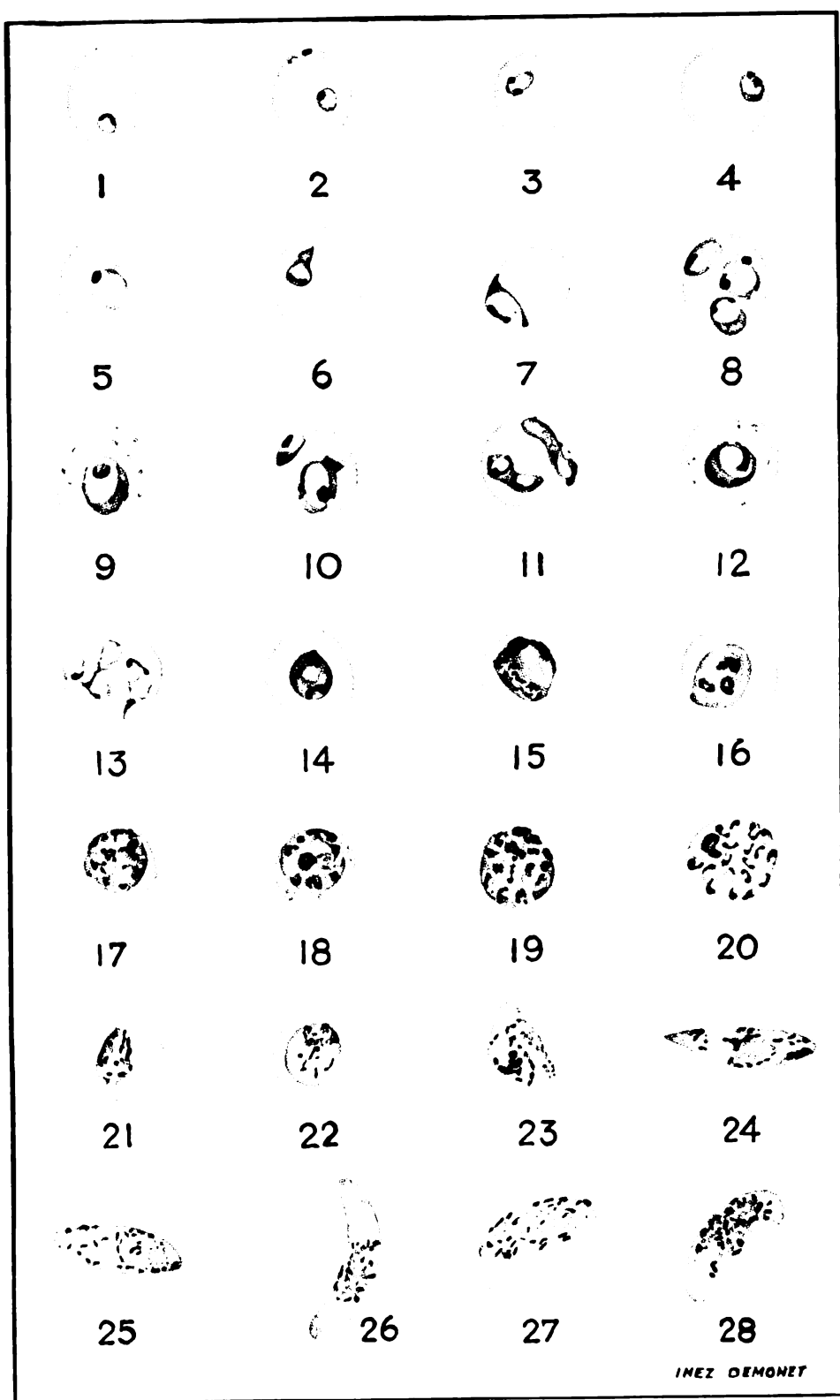


Figure 38 Development of malarial parasite: *Plasmodium falciparum*.

But since Wright's stain is alcohol soluble, the fixing and laking processes must be reversed. First lake the *thick* film by immersing it in distilled water for about 30 seconds, or until the hemoglobin ceases dissolving out. Then stain the entire slide with Wright's stain; the thin film need not be fixed in advance.

d. STAINING SLIDES IN GROUPS. Groups of combined thick and thin preparations can be stained simultaneously with Giemsa's stain. Thin pieces of cardboard the size of a slide label are placed between the slides at the *thin-film end* and the slides are tied together with string in groups of 25. The staining procedure is then followed exactly as recommended for single slides. The portions of the thin films that are covered by the cardboard will not be stained; however, inasmuch as a greater number of parasites tend to occur at the end of the thin smear rather than at the beginning, satisfactorily stained specimens are obtained.

CHAPTER 14

HELMINTHOLOGICAL METHODS

Section I. CLASSIFICATION

466. General

a. The helminths likely to infect the soldier may be classified in three main groups: flukes (trematodes), tapeworms (cestodes), and roundworms (nematodes).

- (1) Adult flukes and tapeworms are usually flat and are grouped in the phylum Platyhelminthes. Adult flukes superficially resemble leeches in appearance although they are in no way related to this group. On the other hand, adult tapeworms are ribbonlike, with a long series of segments set behind a very small head and neck.
- (2) The roundworms (also called threadworms) are grouped in the phylum Nematelminthes. Many of the small forms resemble bits of thread, but in the large forms the worm-like character is more evident.

b. The life cycles of many parasitic helminths are complicated since they frequently involve several hosts. Lack of space prohibits more than a general account of the life cycles in this group. (See tables LX and LXI.) For details of the individual life cycles of these and other parasitic forms, the medical soldier is referred to standard works in the fields.

467. General Considerations for Laboratory Diagnosis

a. Worms living in various parts of the body may cause pathologic changes with the appearance of certain clinical symptoms. Some cases of helminthic infection may be diagnosed by clinical symptoms alone but it is always more reliable if the parasite itself (in any stage—egg, larva, or adult), or a part of the parasite, can be demonstrated. In many cases identity of the adult helminths is difficult to determine, and some of the larval forms are even more difficult. The eggs of the various species, however, are usually sufficiently distinctive to serve as an excellent means of diagnosis in routine laboratory procedure.

b. The life history or life cycle of each parasite governs the type of material to be identified and its source. Most species can be diagnosed by finding their eggs in the host's feces. A brief statement for each species will be given in the following section and

Table LX. *Hosts of human helminths*

FINAL HOST—MAN

Parasite	1st intermediate host	2d intermediate host
Flukes:		
Blood flukes	Snails	None.
Other flukes	Snails	Fish or crustacea; aquatic plants. ¹
Tapeworms:		
Broad fish tapeworm	Copepods (<i>Cyclops</i> , etc.) ..	Fish.
Beef tapeworm	Cattle	None.
Pork tapeworm	Hogs and man ²	None.
Rat tapeworm (<i>H. diminuta</i>) ..	Fleas, beetles, etc.	None.
Roundworms:		
Guinea worm (<i>D. medinensis</i>) ..	Copepods (<i>Cyclops</i> , etc.) ..	None.
Filarioidea	Blood-sucking insects	None.

INTERMEDIATE HOST—MAN

Parasite	Final host
Tapeworms:	
<i>Sparganum</i> and related forms	Dog, cat, etc.
Pork tapeworm (<i>Cysticercus cellulosae</i>) ²	Man.
Hydatid worm (<i>E. granulosus</i>)	Dog.
Roundworms:	
<i>Trichinella spiralis</i>	Rats, hogs, man.

NO INTERMEDIATE HOST

Tapeworms:
Dwarf tapeworm (*H. nana*)

Roundworms:
Pinworms.
Whipworm.
Ascaris.
Hookworm.
Strongyloides.

¹ Serve as equivalent of a 2d intermediate host.

² Man may serve as both intermediate and final host.

grouped according to the medium or site to be considered in laboratory diagnosis. Many of the diagnostic stages are illustrated in figure 39.

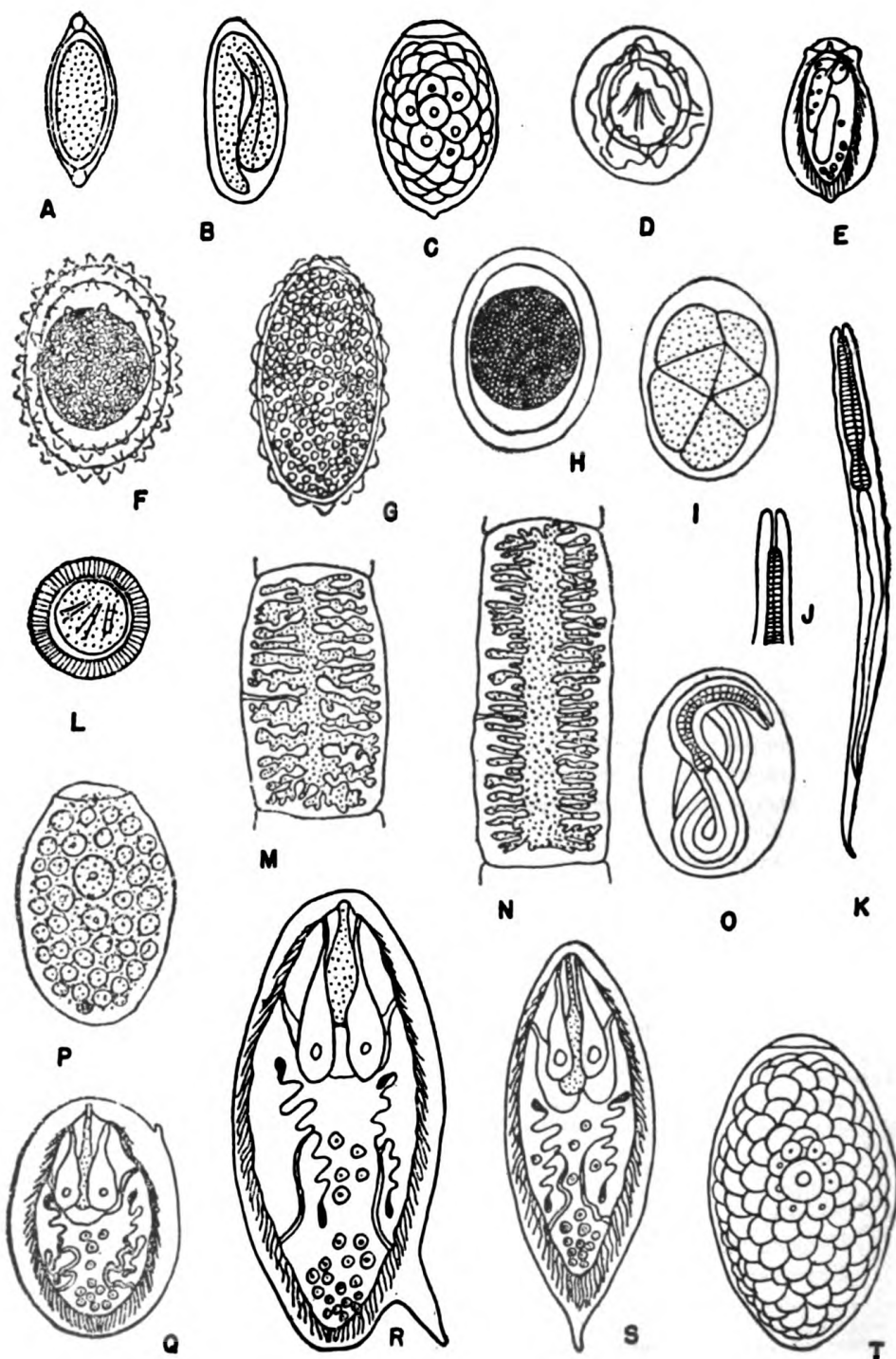


Figure 39 Helminths (diagram of diagnostic material).

Table LXI. Methods of Entry of Helminths into Man

Larvae penetrate skin		Organisms ingested	
Terrestrial	Aquatic	As egg	As encysted larva
By fecal contamination:	by swimming larvae:	By fecal contamination:	In food:
Hookworm.	Blood flukes.	Whipworm.	Broad fish tapeworm.
<i>Strongyloides.</i>		Pinworm.	<i>Trichinella.</i>
<i>Ancylostoma braziliense.</i>		<i>Ascaris.</i>	Liver flukes.
By insect bites:		<i>H. nana.</i>	Intestinal flukes.
All <i>Filarioidea.</i>		Pork tapeworm.	Lung flukes.
		Hydatid worm.	Beef tapeworm.
By accidental contact:			Pork tapeworm.
<i>Sparganum.</i>			<i>Sparganum.</i>
			<i>H. diminuta.</i>
			In water:
			<i>Dracunculus</i> in copepods.

Section II. IMPORTANT PATHOGENIC SPECIES

468. Helminths in Feces

a. MANSON'S BLOOD FLUKE (*SCHISTOSOMA MANSONI*) R, fig. 39). Infection is acquired by swimming or wading in water containing the infective larval stage. These larvae (cercariae) penetrate the skin and enter the blood stream. The adults usually

A, egg of the whipworm (*Trichocephalus trichiurus*); B, egg of the pinworm (*Enterobius vermicularis*); C, egg of the broad fish tapeworm (*Diphyllobothrium latum*); D, egg of the dwarf tapeworm (*Hymenolepis nana*); E, egg of the Chinese liver fluke (*Clonorchis sinensis*); F, G, H, fertilized, unfertilized, and decorticated eggs, respectively, of the large intestinal roundworm (*Ascaris lumbricoides*); I, egg of hookworm (*Necator americanus* or *Ancylostoma duodenale*); J, forepart of rhabditoid larva of hookworm showing long buccal cavity. Compare with short buccal cavity of rhabditoid larva of *Strongyloides stercoralis*; K, L, egg of either of the pork tapeworm (*Taenia solium*) of the beef tapeworm (*Taenia saginata*); M and N, gravid segments of the pork and beef tapeworms, respectively, showing difference in the number of uterine branches (fewer than 15 in pork tapeworm, more than 15 in beef tapeworm); O, egg of hookworm showing fully developed embryo (commonly found in constipated stools of hookworm patients); P, egg of the oriental lung fluke (*Paragonimus westermani*); Q, egg of the oriental blood fluke (*Schistosoma japonicum*); R, egg of Manson's blood fluke (*Schistosoma mansoni*); S, egg of the vesical blood fluke (*Schistosoma hematobium*); T, egg of the sheep liver-fluke (*Fasciola hepatica*) or the large intestinal fluke (*Fasciolopsis buski*). (Approximate magnifications, E X600; M and N, X3; all others X300.)

Figure 39—Continued

inhabit the mesenteric vessels draining the large bowel. This species is distributed in parts of Africa, particularly the Nile delta and a large equatorial section extending east and west to both coasts; it also found in Dutch Guiana, Venezuela, Northern Brazil, Puerto Rico, and several of the lesser Antilles. *Laboratory diagnosis*: eggs (115-175 μ) usually in feces—rarely in urine,—are medium thick-shelled with a very pronounced lateral spine and contain a fully formed miracidium when passed.

b. ORIENTAL BLOOD FLUKE (*SCHISTOSOMA JAPONICUM*) (Q, fig. 39). Infection is acquired by the same means as Manson's blood fluke. Adults of this species also inhabit the mesenteric vessels draining the large bowel. This fluke is confined to the Orient—Central China (principally in the Yangtze Valley) and to a lesser extent South China, Japan, Central Formosa, the Philippines, and the Celebes. *Laboratory diagnosis*: eggs (70-100 x 55-70 μ) in feces have a heavy shell with a small lateral depressed hook, which may look like a knob, and contain a fully formed miracidium.

c. VESICAL FLUKE (*SCHISTOSOMA HAEMOTOBIUM*) (S, fig. 39). Eggs sometimes seen in feces.

d. LARGE INTESTINAL FLUKE (*FASCIOLOPSIS BUSKI*) (T, fig. 39). Infection is acquired by accidental ingestion of infective larval cysts (metacercariae), usually while eating pods, stems, roots, or bulbs of water plants, or in peeling water chestnuts with the teeth. The adults inhabit the duodenal region of the small intestine. The distribution is limited to the Orient; they are found particularly in Chekiang Province of Central China and to a lesser extent in South China and French Indo-China. *Laboratory diagnosis*: eggs (130-140 x 80-85 μ) in feces are commonly ellipsoidal in shape but are irregular both in size and shape, have a medium-thick wall with a small lid or operculum on one end and contain a single fertilized cell surrounded by a mass of yolk cells.

e. SHEEP LIVER FLUKE (*FASCIOLA HEPATICA*) (T, fig. 39). This has a world-wide distribution in sheep and cattle but is uncommon in man. *Laboratory diagnosis*: eggs in feces closely resemble those of *F. buski*.

f. CHINESE LIVER FLUKE (*CLONORCHIS SINENSIS*) (E, fig. 39). Infection is acquired by ingestion of encysted larvae (metacercariae) while eating raw (infected) fresh-water fish. Adults live in the bile passages of the liver and eggs pass down the bile ducts. Distribution is confined to the Sino-Japanese area of Orient; they are particularly common in Kwangtung Province in South-China, northern French Indo-China, Southern Korea, and a few localized areas in Japan. *Laboratory diagnosis*: eggs (28-35 x 12-20 μ) in feces are thick-shelled with pronounced shoulders protecting

the operculum at the smaller end and contain a fully formed miracidium.

g. CAT LIVER FLUKE (*OPISTHORCHIS FELINEUS*). This form is closely related to *C. sinensis* in morphology and life history. It occurs in man along the east coast of the Baltic Sea inland through northern U.S.S.R., particularly in Siberia. It is relatively rare in man in the Orient so that there is little chance of confusing its eggs with those of *C. sinensis*. *Laboratory diagnosis*: eggs (29 x 12 μ) in feces are similar to those of *C. sinensis*; they differ from the latter primarily by being more slender and having an even more pronounced shoulder protecting the operculum.

h. SMALL INTESTINAL FLUKE (*METAGONIMUS YOKOGAWAI*). Man becomes infected by eating uncooked fish containing the metacercaria. The adults live in the small intestine and are comparatively harmless except in very heavy infections. They are commonly found along the Pacific Coast of Asia from South China north and in Japan and Formosa. *Laboratory diagnosis*: eggs (26–28 x 15–17 μ) in feces have thick shell with large operculum without pronounced shoulders and contain living miracidium.

i. SMALL INTESTINAL FLUKE (*HETEROPHYES HETEROPHYES*). This fluke is similar to *M. yokogawai*. Although less common in the Orient, it is found in South and Central China, Korea, Japan, Formosa, and, to a lesser extent, the Philippines; it is quite frequent in the Nile Delta of Egypt. *Laboratory diagnosis*: eggs (28–30 x 15–17 μ) similar to those of *M. yokogawai* but slightly longer.

j. ORIENTAL LUNG FLUKE (*PARAGONIMUS WESTERMANI*). See paragraph 472.

k. BROAD FISH TAPEWORM (*DIPHYLLOBOTHRIUM LATUM*) (C, fig. 39). This tapeworm is acquired by eating raw or insufficiently cooked fresh-water fish infected with the larval form (plerocercoid). The adults inhabit the small intestine, where they lay eggs. Infection is particularly common in Finland and other countries bordering on the Baltic. It is also found in certain places in Siberia, Manchuria, Japan, Russian Turkestan, and Rumania. It has been reported from the Swiss lake regions and has been introduced into the Great Lakes region of the United States and Canada and into northern Florida. *Laboratory diagnosis*: eggs (70 x 45 μ) in feces have a moderately thick shell with a small but discernable operculum and contain an embryo in the early cleavage stages.

l. BEEF (UNARMED) TAPEWORM (*TAENIA SAGINATA*) (L and N, fig. 39). This is the commonest human tapeworm. It is acquired by eating insufficiently cooked beef that contains the larval form

(cysticercus). The adults inhabit the small intestine. Distribution is cosmopolitan. *Laboratory diagnosis*: gravid proglottids (segments) in feces have a central uterine stalk with 15 to 40 lateral branches; eggs (30–40 μ), within the uterus are round or ellipsoidal, have a heavy radially striated shell and contain an embryo with six hooks (onchosphere); eggs rather than proglottids may occur in the feces occasionally, but proglottids can be expelled by using a saline cathartic.

m. PORK (ARMED) TAPEWORM (TAENIA SOLIUM) (L and M, fig. 39). This tapeworm has a comparatively rare but world-wide distribution. Two types of human infection may occur. The more common is with the adult, acquired by accidentally ingesting infective larval forms (cysticercus) with insufficiently cooked infected pork. The adults usually inhabit the small intestine. Less frequently, man serves in place of the intermediate host for this tapeworm by ingesting the eggs. The resulting cysticerci may develop in the skeletal musculature but just as commonly in the brain, where they give rise to a serious condition, often with symptoms of Jacksonian epilepsy. Diagnosis of cysticerci is difficult, and is usually not attempted in the routine laboratory unless the cysts are located in superficial tissues, whence they may be excised and examined. X-ray films are helpful in diagnosing calcified cysts located in vital centers. A history of infection with the adult worm is suggestive since it is possible to ingest the eggs from one's own feces. *Laboratory diagnosis*: proglottids in feces similar to those of *T. saginata* except that there are fewer lateral branches (6–15) on the central uterine stalk and these branches may terminate into dendritic branching; eggs are indistinguishable from those of *T. saginata* and occur in feces under the same circumstances.

n. DWARF TAPEWORM (HYMENOLEPIS NANA) (D, fig. 39). This parasite, worldwide in distribution, is the commonest tapeworm in the southern United States. Infection is acquired by infestation of eggs. The adults inhabit the small intestine. No intermediate host is necessary, and the person harboring the infection can readily reinfect himself by accidentally ingesting eggs evacuated in his own feces. *Laboratory diagnosis*: eggs (37–47 μ) in feces are round or ellipsoidal and have a thin shell within which the small hexacanth larva (onchosphere) appears to be suspended by polar filaments radiating from two opposite poles.

o. RAT TAPEWORM (HYMENOLEPIS DIMINUTA). This is a rare tapeworm of man. Eggs resemble those of *H. nana* but may be distinguished by their larger size and the absence of polar filaments.

p. HUMAN PINWORM OR SEATWORM (*ENTEROBIUS VERMICULARIS*, SYN. *OXYURIS VERMICULARIS*) (B, fig. 39). Infection is acquired by ingestion of embryonated eggs. The eggs are usually embryonated when deposited by the female. The adults live in the cecum, appendix, the adjacent parts of the large and small intestines. The gravid females usually pass out through the anus and deposit eggs on the perianal and perineal folds. Eggs are not commonly deposited in large numbers in the bowel, and in examining for evidence of pinworm infection, ordinary fecal examination will not suffice. The distribution of this infection is world-wide. *Laboratory diagnosis*: eggs (50–55 x 20–30 μ) obtained by perianal scraping (NIH swab, par. 473 g) are thin-shelled, ellipsoidal and flattened on one side and contain an embryo in the advanced morula stage (solid mass of cells) or early wormlike stage; eggs or adult females are occasionally seen in feces.

q. HUMAN WHIPWORM (*TRICHURIS TRICHIURA*, SYN. *TRICHOCEPHALUS TRICHIURUS*) (A, fig. 39). This common intestinal roundworm is acquired by ingestion of embryonated eggs. Adults usually live in the cecum, but may be found in other parts of the large bowel and even in the appendix. There is a world-wide distribution. *Laboratory diagnosis*: eggs (50–55 x 22–24 μ) in feces are barrel-shaped with a double shell, the outer one being yellowish brown; the egg has transparent “plugs” at each end and contains a single large fertilized cell surrounded by yolk.

r. LARGE INTESTINAL ROUNDWORM (*ASCARIS LUMBRICOIDES*) (F, G, and H, fig. 39). The larvae which hatch from these eggs in the small intestine penetrate through the gut wall and migrate via the blood stream to the lungs and from there up the trachea and back down the esophagus to mature in the small intestine. This is one of the commonest worm parasites of man and has a world-wide distribution. *Laboratory diagnosis*: eggs (50–60 x 35–45 μ) in feces are usually broadly oval with a heavy shell surrounded by an outer albuminous, mammillated covering, which may sometimes be lacking; the egg contains a single large fertilized cell and numerous yolk granules; eggs which lack the albuminous mammillated coat are said to be decorticated; unfertilized eggs are longer (88–94 μ) but have a thinner shell, which may or may not have the outer mammillated coat.

s. HOOKWORM (*NECATOR AMERICANUS* OR *ANCYLOSTOMA DUODENALE*) (I, J, and O, fig. 39). Infection is acquired by invasion of infective (filariform) larvae through the skin. The adults usually live attached to the mucosa of the small intestine. It is distributed in warm, moist regions throughout the world. For the most part, however, the American hookworm (*Necator americanus*) is found in the Western Hemisphere and in the middle and

southern two-thirds of Africa; whereas the Old-World hookworm (*Ancylostoma duodenale*) is usually found in Europe and in the northern third of Africa. In Asia, the East Indies, and Australia both species of hookworm are encountered. *Laboratory diagnosis*: eggs (55–65 x 35–40 μ) in the feces have a thin shell and are in the early cleavage stages (4–8 cells) that do not completely fill the egg, leaving a clear space, particularly at the ends.

t. **STRONGYLOIDES STERCORALIS** (K, fig. 39). Infection is acquired by the invasion of infective (filariform) larvae through the skin, or by accidental ingestion of these larvae. In either event the larvae enter the blood stream and reach the alimentary tract indirectly via the respiratory tree. The females usually inhabit the walls of the duodenum and upper jejunum. The eggs are laid and hatch in the tissues, the larvae (rhabditoid larvae) migrating into the intestinal lumen and passing out in the host's feces. Sometimes these larvae, which are normally not infective, may transform into the infective type (the filariform larvae) during their transit in the intestine. These infective larvae may invade the tissues of the lower bowel producing reinfection. The parasite is distributed in warm, moist regions throughout the world. *Laboratory diagnosis*: active rhabditiform larvae (0.4–0.5 mm long) in fresh, cleanly collected feces are presumptive evidence of infection with *Strongyloides*; distinguishing the buccal cavity, which is shorter than the width of the worm at that point, is confirmatory. Further confirmation may be obtained by culturing the material in equal parts of feces and moist charcoal or sterile sand at room temperature to obtain the filariform (infective) larvae in 2 days; these may be isolated by suspending the culture in a thin cloth in warm water, whereupon the larvae will settle down and can be identified (under a microscope) by the notched tail. Repeated examinations are frequently necessary.

469. Helminths in Blood

a. **BANCROFT'S FILARIA (WUCHERERIA BANCROFTI)**. Infection is acquired during the bites of infected mosquitoes. Adults normally inhabit the lymphatic vessels and the lymph nodes, microfilariae probably being deposited in these places and carried into the blood stream. They are distributed throughout the world in tropical and subtropical regions. *Laboratory diagnosis*: sheathed microfilariae (250–300 μ long), in thick or thin blood smears, that are usually most plentiful in peripheral blood from 10 PM to 2 AM.

b. **WUCHERERIA MALAYI**. This form is closely related to *W. bancrofti* and like the latter utilizes a mosquito as the intermediate

host. It is common in many parts of the Dutch East Indies and is also found in India and China. *Laboratory diagnosis*: sheathed microfilariae (170–230 μ long), in thick or thin blood smears, that show nocturnal periodicity.

c. **EYE WORM (LOA LOA)**. Infection is acquired during the bite of the infected mango fly (*Chrysops*). The adults move about in the subcutaneous and deeper cutaneous tissues, microfilariae being discharged into the passages produced during these migrations. This parasite is widely distributed in central West Africa. *Laboratory diagnosis*: sheathed microfiliae (250–300 μ long), in thick or thin blood smears, that show diurnal rather than nocturnal periodicity.

d. **PERSISTENT FILARIA (ACANTHOCEILONEMA PERSTANS)**. Infection is acquired during the bite of the infected “punkie” (*Culicoides austeni*, and possibly *C. grahami*). Adults live in the body cavities and associated tissues, including mesentery, pleural cavity, etc. They are distributed in Africa, South America, and the East Indies. *Laboratory diagnosis*: unsheathed microfilariae (about 200 μ long), in thick or thin blood smear, that show no periodicity.

e. **MANSONELLA OZZARDI**. Human infection is acquired from the bite of the infected sandfly. *Culicoides furens*. The adult lives in the body cavity and the disease is usually asymptomatic. It is found in northern South America, Panama, and the Yucatan peninsula of Mexico and has also been reported from the West Indies. *Laboratory diagnosis*: unsheathed microfilariae (about 185 μ long) in thick or thin blood smears, that show no periodicity.

f. Microfilariae of the pathogenic species inhabiting the blood should be carefully differentiated from the harmless varieties; detailed descriptions are available in medical text books. The technique for the examination of blood is given in chapters 2 and 6.

470. Helminths in Other Tissues

a. **HYDATID WORM (ECHINOCOCCUS GRANULOSUS)**. Hydatid disease is infection with the larval stage of a dog tapeworm (*Echinococcus granulosus*). Human infection is acquired by accidental ingestion of eggs. The larval form (hydatid) develops from the egg and may encyst in various organs, particularly the liver and the lungs. Hydatid disease is found chiefly associated with sheep raising in Iceland, South America, Australia, and South Africa. It is seen throughout Central Europe and the Mediterranean areas of Europe, Africa, and Asia, and has been reported from the United States. *Laboratory diagnosis*: the most accurate

diagnosis of hydatid cysts involves the use of the intradermal reaction (Casoni test.)

b. CYSTICERUS CELLULOSAE (TAENIA SOLIUM). See paragraph 468 m.

c. SPARGANUM. Under the term "sparganosis" is grouped infection with the larval forms (sparganum stages) of certain tapeworms. In most cases the adults are unknown, but the most common sparganum of man (*Sparganum mansoni*) is, in the adult stage, a tapeworm of dogs and cats. The spargana are found in the subcutaneous and ocular tissue of man where, particularly in the latter region, they may produce intense pain and tissue reaction. Infection may be acquired by poulticing an inflamed surface of the body with the infected flesh of a cold-blooded vertebrate (for example, frogs). The great majority of cases of human sparganosis have been reported from the Orient, but sporadic cases have appeared in other parts of the world, including the United States. *Laboratory diagnosis*: this is based on the excision of the spargana from the site of infestation.

d. TRICHINA WORM (TRICHINELLA SPIRALIS). Infection is acquired by eating insufficiently cooked pork that is infected with the encysted larvae. The females deposit larvae in the lymphatics of the duodenum and upper jejunum, perhaps also in the mesenteric veins. Larvae are carried to all parts of the body and migrate and encyst in striated muscles. Infections are distributed chiefly in the United States and Central Europe, rarely reported from parts of Africa and South America. *Laboratory diagnosis*: this usually is based on an intradermal test or a precipitin test, or on recovery of larvae in excised muscle. An eosinophilia rising sharply within a short time after onset of the illness is suggestive.

e. CONVOLUTED FILARIA (ONCHOCERCA VOLVULUS). Infection is acquired during the bite of the blood-sucking black fly (*Simulium*) infected with the larval forms. Adults usually live in tumors in subcutaneous or connective tissues and deposit larvae inside of these nodules; they are distributed in Central Africa, western Guatemala, and southern Mexico. *Laboratory diagnosis*: this is based on demonstration of adults or microfilariae from excised nodules.

f. GUINEA WORM (DRACUNCULUS MEDINENSIS). Infection is acquired by ingestion of infected small crustaceans (*Cyclops*) in raw drinking water. Adults develop in the viscera or in the subcutaneous tissue and, when mature, usually migrate to the subcutaneous tissue of the leg below the knee. A blister develops on the skin just below the head of the worm; after a few days this blister bursts, and on contact with fresh water, the worm deposits

larvae into the water through the ruptured blister. It is common in certain regions of Central Africa north of the equator and the eastern half of India and it is also found in scattered foci in Arabia, Iran, Russian Turkestan, and Afghanistan. *Laboratory diagnosis:* This is based on finding the worm under the skin when local lesions have developed.

g. ANCYLOSTOMA BRAZILIENSE. This species is a tropical hookworm of dogs and cats. The larvae penetrate the superficial layer of the human skin on contact, but since they cannot penetrate the blood vessels, they migrate in the epidermis producing a "creeping eruption." Many human cases of creeping eruption have been reported from the southern United States, but cases are likely to be encountered wherever animals harbor the adult hookworm.

471. Helminths in Urine

a. VESICAL BLOOD FLUKE (SCHISTOSOMA HEMATOBIMUM) (S, fig. 39). Infection is acquired by the same means as with Manson's blood fluke. Adults of this species inhabit the portal blood and vesical plexuses; they are distributed extensively in Africa. *Laboratory diagnosis:* eggs (110–170 x 40–70 μ) in urine, occasionally in feces, are moderately thick-shelled with a pronounced terminal spine and contain a fully developed miracidium.

b. SCHISTOSOMA MANSONI. The eggs are sometimes found in the urine. (See par. 468a.)

472. Helminths in Sputum—Oriental Lung Fluke (*Paragonimus westermani*) (P, fig. 39)

Infection is acquired by consumption of raw crabs and crayfish harboring the metacercariae. The adults are usually found in cysts of the lung, although they occur in other sites. The principal foci are in Korea, Formosa, Japan, and Central China. *Laboratory diagnosis:* eggs (80–120 x 50–60 μ) in sputum (or in feces) are thick-shelled and ellipsoidal-shaped, with a large operculum on one end, and contain a single fertilized cell surrounded by yolk cells.

Section III. METHODS OF EXAMINATION

473. Feces

a. DIRECT SMEAR. This is the oldest, simplest, and most widely applicable method of fecal examination. Use microslide or double cover slip method. (See par. 451a.) For the most part worm infections heavy enough to be of clinical significance can be detected

by direct smears, provided it is a species in which microscopical stages are ordinarily found in the feces.

b. SIMPLE FLOTATION TECHNICS.

(1) Either a solution of salt (sodium chloride) or sugar may be used, and both are generally available. Stock solutions are prepared as follows:

(a) *Salt*. Bring water to boil in the presence of an excess of salt; the supernatant after cooling has a specific gravity of 1.198. This saturated aqueous solution is satisfactory for the ova of hookworm, *Ascaris*, whipworm, and the dwarf tapeworm.

(b) *Sugar*. Two pounds (910 gm) of sugar to 1,125 ml of water, heated to get the sugar in solution, produces a levitating fluid with a specific gravity of about 1.200. This solution is satisfactory for the ova mentioned previously, as well as for protozoan cysts; it will sometimes raise the rhabditiform larvae of *Strongyloides* and the ova of *Schistosoma mansoni*, although distortion may be expected in the latter cases.

(2) Fresh feces may be mixed with either fluid, and after standing for 10 or 20 minutes the eggs will be on the surface and should be transferred to a slide. This may be done with a large (10-15 mm) wire loop, or by allowing them to float up against a slide or cover glass placed over the lips of the container which has been filled with the fecal mixture so that the fluid surface will contact the cover glass or slide.

c. ZINC SULFATE CENTRIFUGATION FLOTATION TECHNIC. This is nearly as effective for floating ova, and for practical purposes in a diagnostic laboratory it may be considered just as effective. It has the advantage that it may float trematode eggs also. (See par. 451b.)

d. ACID-ETHER TECHNIC has been shown to be efficient and practicable for the demonstration of *S. mansoni* and *S. japonicum* eggs. About 1 gm of fecal material (about the size of a pea) is thoroughly emulsified in 5 ml of 40 percent HCl (40 ml concentrated HCl diluted to 100 ml) in a small vial. The material is filtered through two layers of moist gauze stretched over the top of a 50 mm funnel into a 15 ml centrifuge tube. An equal quantity of ether is added and the tube is stoppered with a gloved finger and shaken thoroughly. It is then centrifuged for 1 minute at 1,500 rpm. On removal from the centrifuge, the debris floating at the acid-ether junction is loosened by ringing with a clean applicator and the acid and ether layers are rapidly poured off

and discarded. The same applicator is then used to stir the sediment in the few drops of fluid remaining; the sediment is then transferred to a slide and examined under a cover slip.

e. A FIELD METHOD FOR THE CONCENTRATION OF SCHISTOSOMA AND OTHER HELMINTH OVA, HELMINTH LARVAE AND PROTOZOAN CYSTS.

- (1) Emulsify 5 gm of fecal material in 10 percent ethyl alcohol and strain through 2 to 4 layers of wet gauze into a 200 ml conical urinalysis flask and fill flask with 10 percent ethyl alcohol.
- (2) Allow to sediment for 1 hour, 45 minutes, and 30 minutes, respectively, decanting each time and replacing with fresh solution. After final sedimentation, decant and examine residual sediment.

f. SODIUM SULFATE ACID-ETHER TRITON TECHNIC FOR CONCENTRATION OF HELMINTH AND SCHISTOSOMA OVA.

- (1) Emulsify 2 gm of fecal material in about 15 ml solution of equal parts of 40 percent hydrochloric acid (40 ml concentrated HCl diluted to 100 ml with water) and sodium sulfate adjusted to specific gravity of 1.08 (concentrated solution of sodium sulfate adjusted to correct specific gravity) and strain through 2 layers of wet gauze.
- (2) Centrifuge at 2,000 rpm for 1 minute in conical centrifuge tubes. Pour off supernatant and wash twice in HCl-sodium sulfate, centrifuging and pouring off supernatant each time.
- (3) After last wash, add 5 ml HCl-sodium sulfate solution, 3 drops of Titron X-30 and 5 ml ether. Invert, shake vigorously and centrifuge for 1 minute at 2,000 rpm.
- (4) Break fat ring with applicator, pour off supernatant, add a few drops of physiological saline solution and examine sediment under the low power objective.

g. NIH (National Institute of Health) ANAL SWAB. In pinworm infection, eggs are more commonly deposited in the perianal folds than in the feces. Therefore, swabbing and mild scraping of these parts yield eggs even when the feces are negative. The NIH anal swab is simple and efficient. It consists of a piece of cellophane (about 25 mm square) held on the end of a glass rod by a rubber band (clippings from hemocytometer tubing will do). The other end of the rod is run through a rubber stopper, thus providing a convenient handle; the stopper also serves to hold the swab in a glass tube in which it may be conveniently transported. (See fig. 40.)

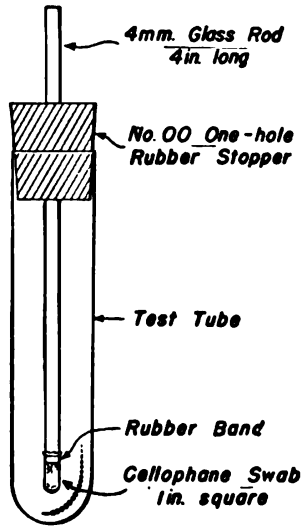


Figure 40. NIH anal swab

A suitable swab may be improvised from standard supply items such as the glass vial used for collecting fecal specimens and a cork in which a hole has been bored. Cellophane from jackets of cigarette packs may be used if otherwise unavailable. The cellophane-covered tip should be stroked over the anal opening and outward over the perianal folds; this is best done in the morning before the patient has bathed or had a bowel movement. For examination the dry cellophane is removed with forceps and mounted with water on a glass slide.

h. GRAHAM SWAB. In this, one holds a piece of scotch tape, sticky side out, over the end of a tongue depressor or blunt forceps, and strokes it over the anal opening and perianal folds in the same manner. The tape may then be mounted, by its own adhesive surface, on a slide and transported to the laboratory for examination.

474. Tapeworm Proglottids

The following method is used to differentiate proglottids (segments) of beef or pork tapeworms passed in the feces:

a. Clean and relax the unfixed proglottids in warm physiologic saline: thereafter they may be soaked in 5 percent warm acetic acid, though this is not essential.

b. Place the specimen between two glass slides and press it flat.

c. Hold it up to a strong light (so that the light shines through it), and count the number of lateral branches of the uterus. (See par. 468 *l* and 468, *m*.) (Since these lateral branches subdivide, they are to be counted where they arise from the main part of the uterus.)

475. Blood

Thick films are useful in demonstrating microfilariae. For details of this technic see paragraph 463. Often microfilariae may be seen more readily by their activity in a fresh drop of blood. (See ch. 2).

476. Other Tissues

Examination of a spinal fluid, of material aspirated from lymph nodes, or of other superficial tissues, and of excised pieces of skin or muscle should usually be performed by the medical officer in charge. Details of these technics are available in standard texts.

477. Urine

a. In cases of heavy infection with the vesical blood fluke, eggs may be found in the urine, particularly in the last portion passed. The technic follows:

- (1) Have the patient pass urine into a urinalysis glass. (This should include the last portion of urine voided.)
- (2) Let the material settle for 15 to 20 minutes.
- (3) Take up a small portion of the sediment in a pipette and place it on a glass slide.
- (4) Examine the sediment under the microscope.

b. In cases of light infection this procedure may yield negative results. It is then necessary to centrifuge a representative portion of the urine for 1 or 2 minutes and to examine the sediment under a microscope.

478. Sputum

In many cases of suspected helminthic infection of the respiratory passage, examination of the sputum is necessary. The technic follows:

- a.* Have the patient rinse his mouth thoroughly with diluted hydrogen peroxide.
- b.* Have the patient raise sputum into a clean jar.
- c.* Transfer small bits of sputum, particularly blood-flecked portions, to a glass slide.
- d.* Examine the preparation under a microscope.

479. Shipment of Specimens (AR 40-310)

a. From time to time it is necessary to send specimens to other laboratories, either for identification or further study. In all such

cases complete notes should accompany the material and should include such data as locality, host, date, collector's name, number of specimens obtained, condition of the specimens, tissue, organ or medium from which recovered, and any other pertinent information. Material may be preserved as indicated below.

- (1) *Eggs.* Feces containing ova may be diluted with water and agitated until an even mixture is obtained. To this mixture is added an equal volume of 10 percent formalin heated to 80° C. This will fix any nematode larvae, ova or protozoan cysts.
- (2) *Larvae and adults.* Larvae and adult helminths should first be shaken in physiologic saline solution. This cleans and relaxes the specimens. They are then fixed by adding an equal volume of hot 5 percent formalin or 70 percent alcohol to the solution.
- (3) *Pathologic tissues* (SR 40-410-10). Pathologic tissues may be fixed in 10 percent formalin or, if it is available, Zenker's fluid. If Zenker's solution is used, after 4 to 24 hours fixation (depending on the size of the specimen) the tissue should be washed in water overnight and then for 1 to 4 hours each in 30 percent and 50 percent alcohol, after which it is stored in 70 percent alcohol.
- (4) *Intermediate hosts.* Almost any intermediate host except adult insects may be fixed and stored in 70 percent alcohol (or 5 percent formalin). A small screw-neck bottle (vial type) is ideal for this purpose. Adult insects should be killed with chloroform or ether fumes or in a cyanide tube. They may be stored and transported between loose layers of tissue paper or lens paper in pill boxes. If snails are collected for identification with no intent of preserving their parasites, they are most conveniently preserved by drying.

b. Specimens should always be carefully packed to avoid breaking or spilling of the contents. Containers should be filled to the top with the preserving liquid to avoid breaking of the specimens if the package is roughly handled. Caps must be sealed with paraffin, sealing wax, or scotch tape. Jars and glass vials packed separately in a box with excelsior, shredded paper or cotton, and marked "Fragile," will usually survive. A double mailing container should be used when the contents are liquid.

CHAPTER 15

ENTOMOLOGICAL METHODS

Section I.

DISTRIBUTION AND CLASSIFICATION OF ARTHROPODS

480. General

a. Medical entomology is the study of arthropods (insects and insect-like animals) and their relation to human disease and discomfort. These arthropods may be associated with a number of diseases of importance to troops in garrison, camp, or campaign. The principal disease relation of arthropods is that of transmission—for example, malaria and yellow fever by certain mosquitoes, plague by fleas, and typhus by lice. Also of importance are the arthropods that cause disease or injury directly by their bites, stings, or actual invasion of tissue. Thus, the itch-mite of man invades the human skin and produces a severe irritation. This disease, known as scabies or itch, caused thousands of men to be admitted to hospitals during World War I. When they occur in large numbers, many arthropods, such as common house-flies and mosquitoes, cause discomfort to troops.

b. Owing to the widespread distribution of arthropods and to their close association with troops, it is necessary that Army personnel be prepared to determine whether the species present are likely to be of medical importance. This may entail collection and tentative identification of representative forms, and the forwarding of specimens likely to be important to central laboratories for positive identification. The medical technician is not expected to know the various species by their scientific names, but if called on to send in a representative sampling of mosquitoes, he should be able to send mosquitoes, and not a variety of small beetles, flies, midges, fleas, moths, etc. It is the purpose of this section to familiarize the technician with the medical importance of various arthropods and to aid him in identifying various groups. The use of a hand lens greatly aids the technician.

c.

- (1) Arthropods have external skeletons formed of a substance called "chitin." With exception of certain larval forms, these animals are characterized by having jointed legs. In common examples such as insects, the various segments of the legs are named as follows: the coxa and

trochanter are two very short segments anchoring the leg to the body; the femur is the first conspicuous segment; next comes the tibia, and finally the tarsus, consisting of one to five smaller segments.

- (2) Many arthropods also have jointed bodies divided into definite regions. Thus the insects have a head, thorax, and abdomen. The head is typically supplied with eyes, a pair of antennae or "feeler," and complicated mouth-parts. On each side of the mouth are one or more appendages called palpi (singular: palp). The mouth parts may be simple for chewing, lapping or both, or they may be built in the form of a sucking tube, which in many forms, such as the mosquito, is adapted for piercing plant and animal tissues.
- (3) The thorax contains most of the muscles of locomotion and is usually rigid in order to support the legs and wings. The abdomen is typically jointed, each region thus demarcated being known as an "abdominal segment." The terminal segments are modified to form the external sex organs. In some forms, the tip of the abdomen contains a stinging apparatus (bees, wasps).
- (4) Most arthropod classification is based on the study of these various external anatomic regions, including specialized hairs, bristles, scales, or other structures that may be found on certain of the parts. The student should remember that in some groups of these animals there has been a fusion of adjacent structures; thus in spiders the head and thorax are combined, in ticks and mites all the parts are merged into a simple saclike unit, in sucking lice there is only a single tarsal joint. These variations will be brought out in the following consideration of individual groups of arthropods.

481. Classification

As an army is divided into corps, divisions, brigades, regiments, battalions, companies, squads, and individual men, animals are grouped into phyla, classes, order, families, genera, and species. The phylum Arthropoda is divided into several classes, four of which are Insecta (insects). Arachnida (ticks, mites, spiders, scorpions, etc.). Myriapoda (centipedes and millipedes), and Crustacea (crayfish, shrimp, etc.). These classes are further divided until single species are reached. For example, the yellow-fever mosquito is classified as follows:

Phylum—Arthropoda
 Class—Insecta
 Order—Diptera
 Family—Culicidae
 Genus—*Aedes*
 Species—*aegypti*

The scientific name of an animal is a combination of the names of the genus and the species, usually printed in italics, the genus capitalized, the species with a small letter. Thus, the scientific name of the yellow-fever mosquito is *Aedes aegypti*.

Section II. ARTHROPODS OF MEDICAL IMPORTANCE

482. Procedure in Identification of Specimens

a. Since all members of any of the above groups possess certain common characteristics that differentiate them from the other classes of arthropods, it is possible to construct "classification keys." A working example of such a key follows:

If the specimen has—

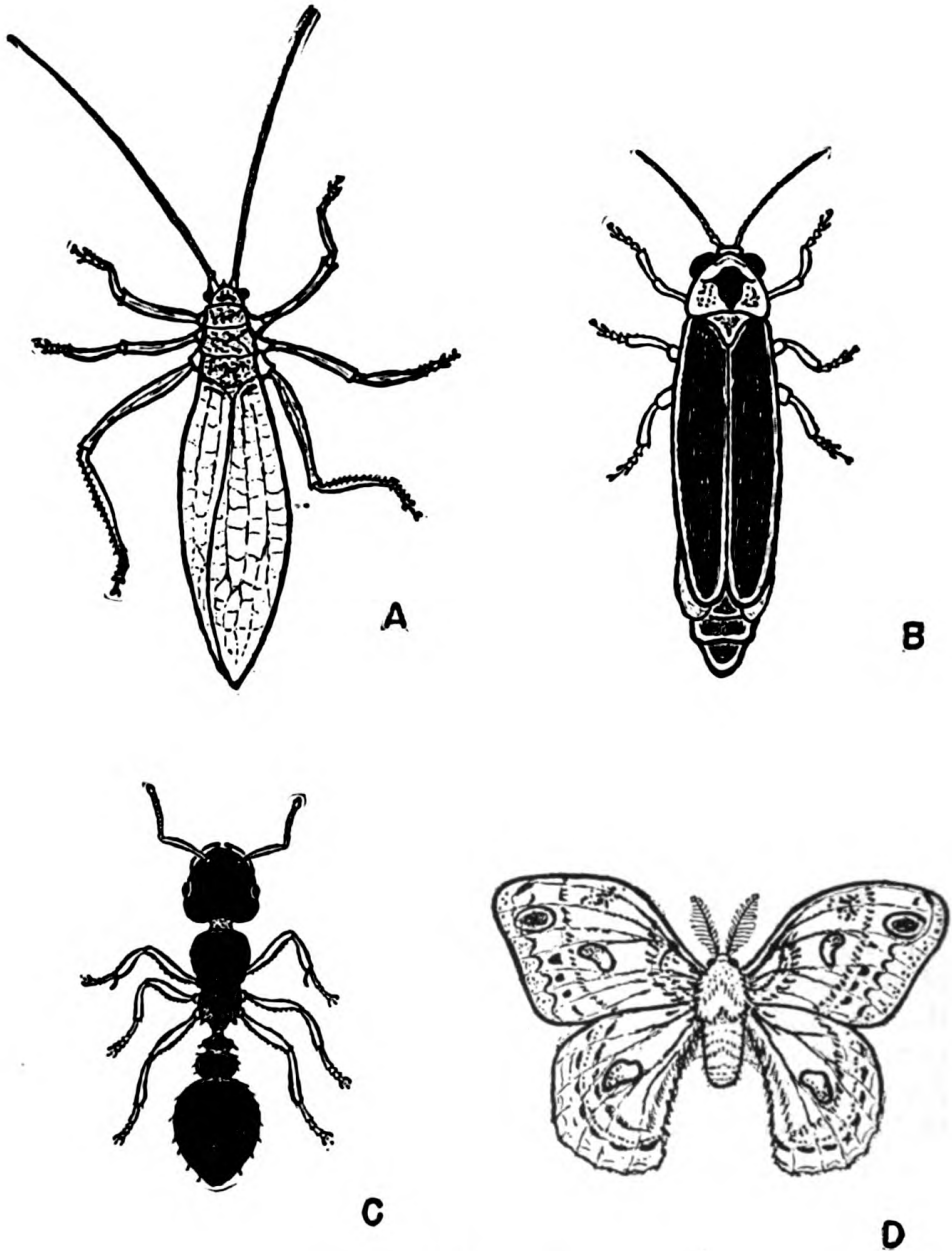
- Three pairs of legs (fig. 41) Insecta (par. 491)
- Four pairs of legs (fig. 44) Arachnida (par. 485)
- Five or more pairs of legs:
 - Lives in water (fig. 42) (exceptions, for example, sowbugs) Crustacea (par. 483)
 - Lives on land (fig. 43) Myriapoda (par. 484)

b. An examination of the above will reveal that identification of specimens is not difficult. If by running the specimen through the key it should prove to be an arachnid, it is necessary only to turn to the section on Arachnida where a key to this class will be found. By subjecting the specimen to several successive keys, a tentative identification can be made.

c. The key aids but does not give final proof of identification. Since in the insect class alone there are thousands of species, it is obvious that the keys and outlines in this manual will not serve to identify all specimens. For more satisfactory identification this manual should be supplemented by standard texts, and for authoritative identification specimens should be transmitted to entomologic centers, such as the Army Medical Service Graduate School, Washington, D.C.

483. Crustacea

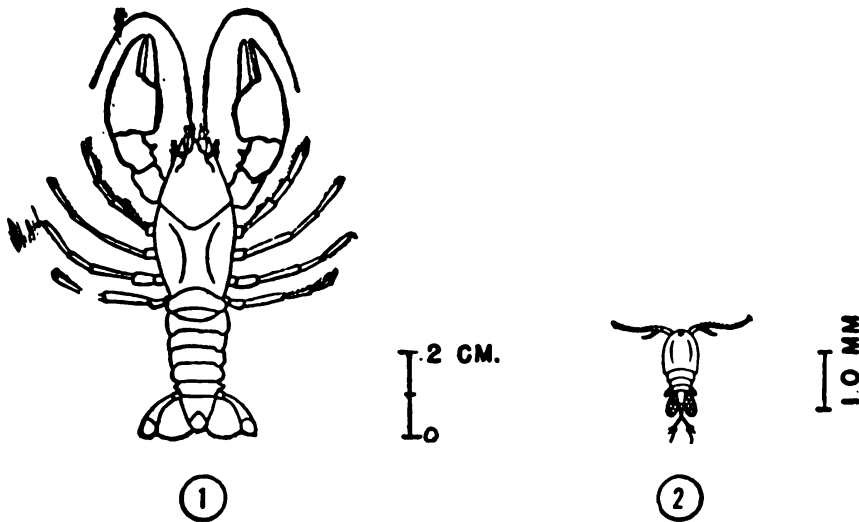
Crustaceans (crayfish, shrimp, etc.) are of little importance to the soldier, but are worthy of mention because a few species are associated with human disease. Several species serve as interme-



A. Katydid (*Orthoptera*).
 B. Firefly beetle (*Coleoptera*).
 C. Ant (*Hymenoptera*).
 D. Moth (*Lepidoptera*).

Figure 41. Typical insects.

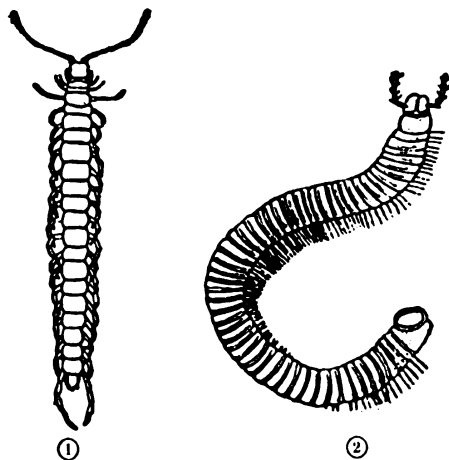
diate hosts in transmitting certain parasites. Two typical crustacea are illustrated in figure 42, one of which (*Cyclops*) is the intermediate host of the guinea worm.



① Crayfish (a crustacean). ② Cyclops.
Figure 42. Typical Crustacea.

484. Myriapoda

Myriapods include the centipedes (Chilopods) (fig. 43①) and the millipedes (Diplopoda) (fig. 43②). They may be differentiated by the number of legs on each body segment, centipedes possessing one pair of legs per segment, whereas millipedes have two pairs on each segment. Only the centipedes are of interest from a medical viewpoint. In this group almost every species possesses a pair of poison fangs, which, in certain forms, are sufficiently sharp and strong to make it possible for them to penetrate the human skin. Although no deaths have been recorded from the bites of centipedes, painful injury can be inflicted. Species of three genera, *Scolopendra*, *Geophilus*, and *Lithobius*, have been found capable of producing injury.



① Centipede. ② Millipede.
Figure 43. Typical myriapods.

485. Arachnida

The class Arachnida is important because it contains many species that serve as transmitters of disease and several species that cause disease in the human body. Following is a key to separate the more important groups:

a. Abdomen not divided into segments:

Body divided by constriction into two main parts

(fig. 44) Spiders (par. 486)

Body not divided by such a constriction (most types parasitic):

Minute species (usually smaller than a pinhead in size,

body hairy) (fig. 45) Mites (par. 487)

Medium-sized species (usually larger than a pinhead in

size, body not hairy) (fig. 46) Ticks (par. 488)

b. Abdomen divided into segments:

With spine at tip of tail (fig. 49 ①) Scorpions (par. 489)

Without spine at tip of tail (fig. 49 ②) Whip scorpions (par. 490)

486. Spiders

Although all spiders produce venom, only a few possess fangs sufficiently powerful to pierce the human skin. Of the most importance to the soldier in the United States is the "black-widow" spider, *Latrodectus mactans*. (See fig. 44.) This is a shiny, medium-sized black spider that can usually be distinguished by the reddish hour-glass marking on the underside of its abdomen. It may be found in grass, shrubs, rock piles, outhouses, and privies. Its bite produces severe symptoms and, in some cases, death. Other equally dangerous species of *Latrodectus* occur in other parts of the world. Tarantulas (co-called) also appear ferocious, but compared to the black-widow spider, the bite is mild.

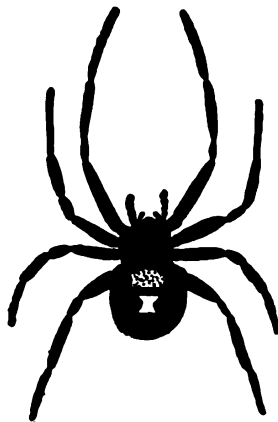


Figure 44. Black widow spider (an arachnid). Female, ventral view.

487. Mites

Most species of mites (fig. 45) are extremely small, many being barely visible to the naked eye. In general, only three forms are of medical importance.

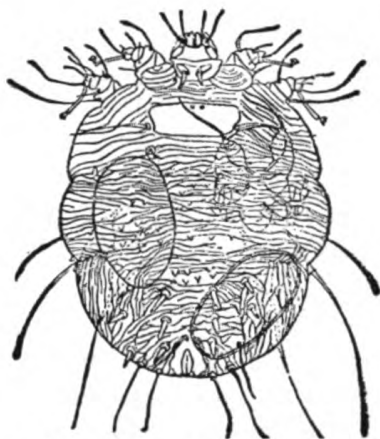


Figure 45. Itch mite of man.

a. Sarcoptes scabiei, the common itch mite of man often assumes medical importance in localities where many people are forced to live together under unhygienic conditions. The skin disease that it produces, scabies, is frequently found in troops. When the mites attack, they usually invade the skin between the fingers, and spread to other parts of the body. The females burrow into the skin and lay eggs in the tunnels made during their migrations. The intense itching that accompanies the disease results in scratching and secondary infection.

b. There is also a widely distributed family of mites the adults of which (with four pairs of legs) are found in soil or on plants. The larvae, however, (with only three pairs of legs) are parasitic on various animals. Two groups of these larvae affect man:

- (1) *American chiggers or redbugs.* The common species is the larva of *Eutrombicula alfreddugesi*. It is also known as *E. irritans* or *Trombicula irritans*. These tiny mites, hardly larger than a pinpoint and bright red, are very numerous during warm weather in fields and woods and in rough growths of shrubs, grasses, and weeds. Although their normal hosts are various animals, including reptiles, troops may become severely infested. The mites crawl beneath the clothing and remain attached to the skin for several days. Their bites produce an extremely intense itching, which may last a week or longer. Their food is not blood, but lymph. Contrary to popular belief

they do not burrow into the skin. Certain repellants applied to clothing around openings, such as the bottom of trousers, fly, wristcuffs, neckbands, and buttoned shirt fronts are effective in preventing attacks. Chiggers may be confused with the larvae of ticks (seed-ticks). Although both are minute and have only three pairs of legs, chiggers are smaller than tick larvae and have rather conspicuous hairs, which are lacking in the latter.

- (2) Larval mites, similar in habits to the American chigger and belonging mostly to the genus *Trombicula*, are frequent in many parts of the world and may cause great annoyance. In Japan and Formosa the mite (*Trombicula akamushi*) transmit the highly fatal Japanese river fever, usually called "*tsutsugamushi disease*." It is also known as "*scrub typhus*," "*tropical typhus*," and "*mite-borne typhus*" in the Philippines, Malay Peninsula, Burma, Sumatra, New Guinea, Northern Queensland (Australia) and elsewhere in the Asiatic-Pacific area. Since these forms of the disease are all transmitted by certain species of trombiculid mites, it is easy to understand how important these almost invisible pests are. Rodents act as reservoir hosts.

488. Ticks

a. The ticks are chiefly important as transmitters of serious diseases, but they are also capable of inflicting bites that are slow to heal and may result in systemic disturbances as well as in

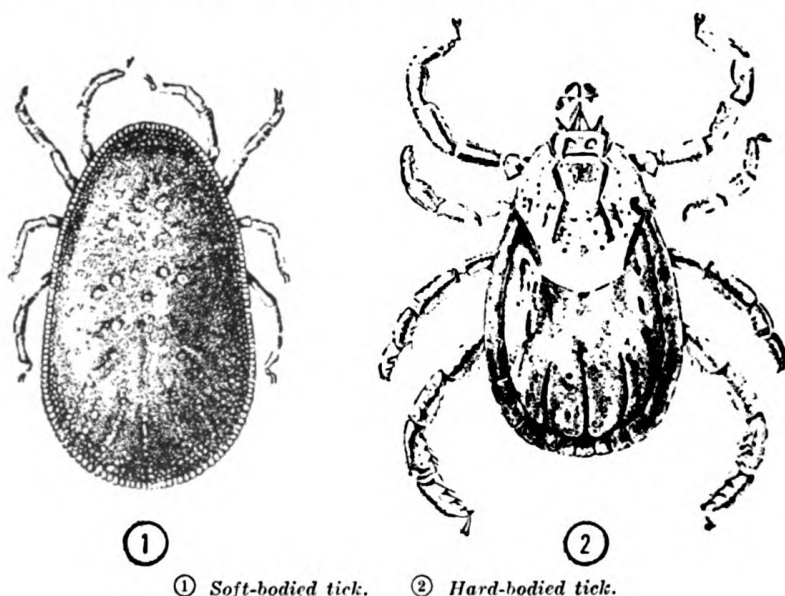


Figure 46. Ticks.

intense local irritation. Ticks have a widespread occurrence, in the tropics and subtropics as well as in temperate regions. For purposes of general classification they are divided into two groups—the soft-bodied ticks (family Argasidae) and the hard-bodied ticks (Ixodidae). These may be distinguished according to the following key:

Head concealed beneath front margin of body (no shield on upper surface of body) (fig. 46 ①) Soft-bodied ticks
Head not concealed beneath front margin of body (shield present on upper surface of body) (fig. 46 ②) Hard-bodied ticks

b. SOFT-BODIED TICKS, ARGASIDAE (fig. 46 ①).

- (1) Ticks of this group, unlike the hard-bodied ticks (Ixodidae), do not remain attached to their hosts for long periods, but feed quickly and then return to their hiding places in cracks and crevices, in houses and caves, under stones, in rodent burrows, etc.
- (2) The genera *Argas* and *Ornithodoros* contain the commonly encountered species. When viewed laterally with a hand lens those of the genus *Argas* are seen to have a fine peripheral line separating the dorsal and ventral surfaces of the body. This line is absent in the genus *Ornithodoros*, in which are found practically all the species of the medically important soft ticks.
- (3) Several species of *Ornithodoros* transmit relapsing fever from animals or human carriers to other animals or to man. The tick acquires the spirochetes (*Borrelia duttoni* and other species) when it takes a blood meal and then infects man or animal at a later feeding. Transovarial infection may occur—that is, the spirochetes are transmitted from adult tick to offspring through the egg. Rodents usually serve as reservoirs of the disease. The ticks principally concerned are *O. tholozani* in Central Asia, *O. moubata* and *O. erraticus* in Africa, *O. rudis (venezuelensis)* in northern South America and Panama, *O. turicata* in Mexico and the southwestern United States and *O. hermsi* in California and certain other western states.

c. HARD-BODIED TICKS, IXODIDAE (fig. 46 ②).

- (1) These ticks, in contrast to the soft-bodied ticks, remain attached to their hosts and feed for long periods of time. They are far more widely distributed, being well represented in tropical, subtropical, and temperate regions. Of the several genera, *Dermacentor* is the most important. Others of importance are *Amblyomma*, *Rhipicephalus*, *Haemaphysalis*, and *Ixodes*.

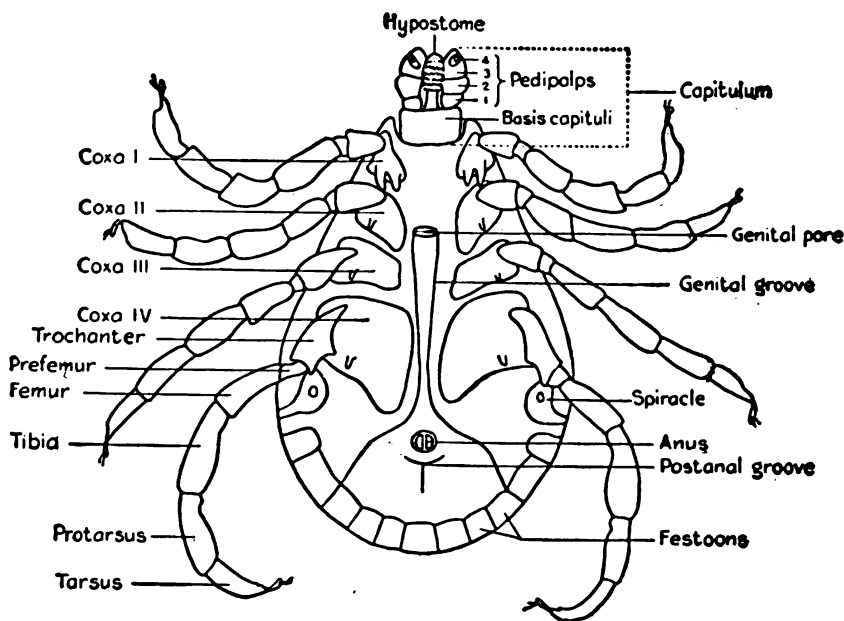


Figure 47. Ventral view of male tick (*Dermacentor andersoni*) showing anatomic structures.

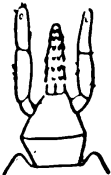
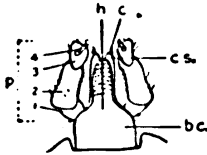
IXODES	HAEMAPHYSALIS	BOOPHILUS
		
AMBLYOMMA	RHIPICEPHALUS	DERMACENTOR
		

Figure 48. Mouth parts of various genera of hard ticks (ventral view), b.c. basis capituli; c., chelicera; c.s. sheath of chelicera; h., hypostome (anchors the tick to host); p., palp.

- (2) Hard-bodied ticks transmit various human diseases, the most important of which are Rocky Mountain spotted fever and tularemia. Rocky Mountain spotted fever, an acute infectious disease having a high mortality, is caused by a rickettsial organism (genus: *Rickettsia*). In the western part of the United States the disease is transmitted by the Rocky Mountain wood-tick, *Dermacentor andersoni*, whereas in the eastern United States the

American dog tick, *D. variabilis* is the chief vector. *Amblyomma americanum* is a vector in the American southwest. The North American rabbit tick, *Haemaphysalis leporis-palustris*, is important in transmitting the disease among rabbits. Tularemia (rabbit fever), caused by *Pasteurella tularensis*, may be transmitted to man by the bites of *D. variabilis* and *D. andersoni*, whereas the rabbit tick is important in transmitting the disease among rabbits. Other rickettsial diseases such as Colombian and Brazilian spotted fever, boutonneuse fever along the Mediterranean, and South African tick-bite fever are transmitted by species of Ixodid ticks.

d.

- (1) In addition to transmitting diseases, many ticks may inflict injury by their bites alone. In the warmer regions of the Americas the immature stages of *Amblyomma americanum* or *A. cajennense*, or both, are abundant during certain seasons. They attack man readily and are a great annoyance to those engaged in outdoor activities.
- (2) The bites of the females of *D. andersoni* and *D. variabilis* and certain species of *Ixodes* are in rather rare instances capable of causing tick paralysis in man and animals. The exact mechanism by which paralysis is effected is not known, but it is generally considered to be due to a toxin. Death may result but it can usually be prevented by early discovery and removal of the tick.

e. At times the technician may encounter parasites that resemble ticks but that have only three pairs of legs. These are usually larval stages of ticks or mites. A few rare insects also have similar characteristics.

489. Scorpions

Scorpions (fig. 49①) may be dangerous to man because some are capable of stinging with a spine that is located at the tip of the tail (abdomen). Many small species are harmless because they are not able to pierce the human skin. Some species of the genus *Centruroides* are very important. In the city of Durango in northern Mexico, *C. suffusus*, causes an average of 50 deaths per year. The common species of the southern United States are generally capable of producing only a painful sting. Of interest to soldiers is the fact that some scorpions crawl into shoes during the night.

490. Whip Scorpions

Whip scorpions (fig. 49©) are ferocious in appearance but are entirely unimportant from a medical standpoint. They are of interest to soldiers because they may be confused with true scorpions.

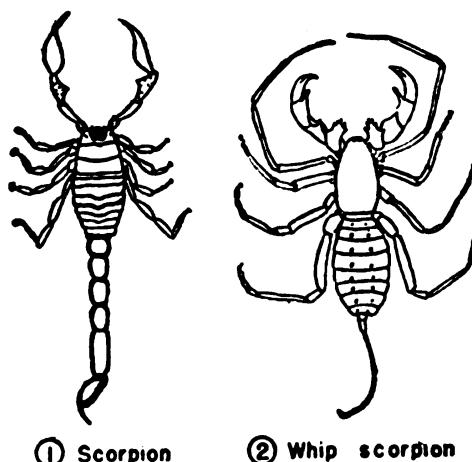


Figure 49. Comparison of common scorpion with the whip scorpion.

491. Insecta

The class Insecta contains many species of medical importance. In this group are included mosquitoes, lice, fleas, bedbugs, etc. The following outline will serve as a guide in classifying the more important orders; those orders of greatest medical importance are marked with an asterisk. (For more complete information and keys the reader is referred to standard entomologic texts):

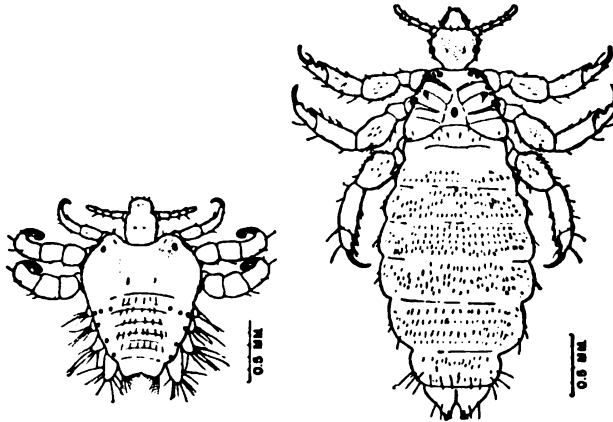
Insect order	Common name or names	Figure
Anoplura*	Lice	50
Coleoptera	Beetles and weevils	41
Diptera*	Flies, mosquitoes, midges, etc.	51, 53, 55
Hemiptera*	Bedbugs, blood sucking conenoses or kissing-bugs, etc.	58, 59
Hymenoptera	Ants, bees, wasps, etc.	41
Lepidoptera	Butterflies, moths, skippers, etc.	41
Orthoptera	Roaches, crickets, grasshoppers, etc.	41
Siphonaptera*	Fleas	60

* Only these groups of marked medical importance will be considered further. The other groups are listed merely to aid the technician in avoiding confusion with the more important species. It should be kept in mind, however, that this list is far from complete.

492. Anoplura (Lice)

There are two species of lice that infest man, namely, the head and body lice (*Pediculus humanus*) and the crab louse (*Phthirus*

pubis). The head louse and body louse are two varieties of the same species, and are called *P. humanus* var. *capitis* and *P. humanus* var. *corporis*, respectively. The head and body lice are easily differentiated from the crab lice by the length of the body in proportion to its width. Both varieties of *P. humanus* are about three times as long as they are broad, whereas crab lice are as broad as they are long. (See fig. 50.)



① Crab louse. ② Body louse.
Figure 50. Typical lice.

a. The head louse lives among the hairs of the head and attaches its eggs (nits) near the base of the hairs by means of a glue formed in a special gland. The hairs around the ears and back of the head are most frequently used by lice as sites for depositing eggs.

b. The body louse is found principally on the inner surface of the under clothing, especially along seams, where eggs are securely attached to the cloth fibers. In general, body lice migrate from the seams for feeding. They take blood several times each day.

c. Man is affected by the head and body louse in two ways—by the mechanical effect of the bites and by transmission of pathogenic organisms. The bites produce minute hemorrhagic spots that are accompanied by irritation and intense itching. These cause scratching and may result in secondary infection.

d. The infectious diseases known to be transmitted by human lice are typhus, trench fever, and one form of relapsing fever. European or epidemic typhus resulted in many deaths among soldiers in the European armies in World War I. It is caused by a rickettsial organism, *Rickettsia prowazeki*, which is transmitted by head or body lice. Transmission is accomplished by deposition of the louse's feces on the injured skin, by crushing the insect against the skin, and possibly by its bite. Trench fever is caused

by an unknown agent, possibly a *Rickettsia*, and is transmitted by the bite or feces of the body louse. Relapsing fever, caused by the spirochete *Borrelia recurrentis*, is transmitted by lice when infective lice are crushed on the skin. The disease is not transmitted by the bite.

e. The crab louse infests chiefly the hairs of the pubic region, but occasionally is found in the axilla, beard, eyebrows, and eyelashes. It does not transmit disease.

493. Diptera (Flies, Mosquitoes, etc.)

a. The order Diptera contains many species that serve to transmit disease and to cause disease. Besides mosquitoes that transmit malaria and tsetse flies that carry sleeping sickness there are many other mosquitoes and biting flies that convey disease. Many species live and breed in close contact with Army personnel and so may spread dysentery.

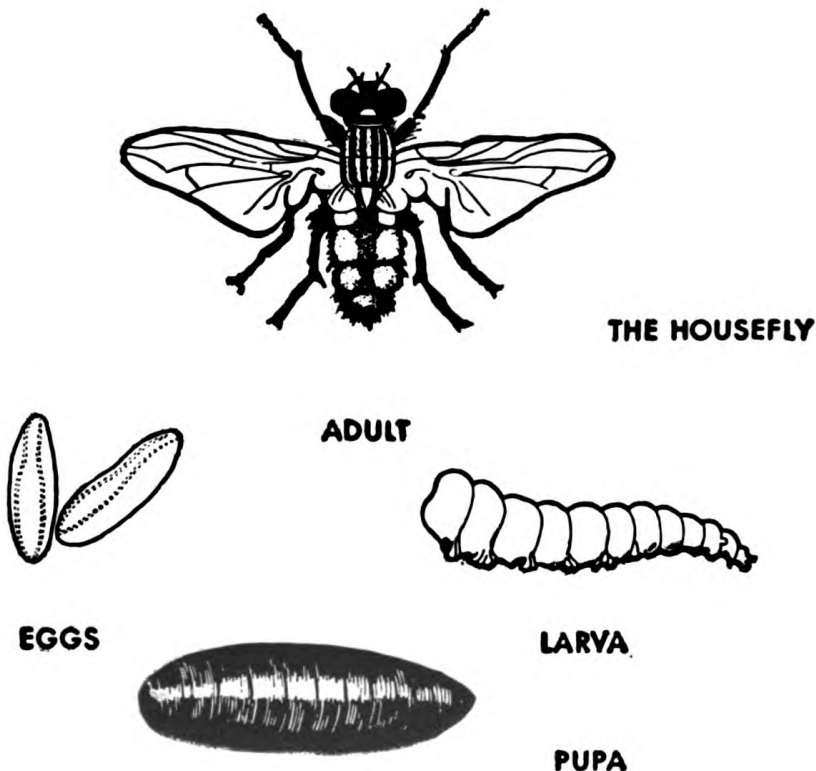


Figure 51. Stages in development of housefly.

b. In their life cycles, flies pass through egg, larval, pupal, and adult stages. (See fig. 51.) The larvae or maggots of some species gain entrance to wounds of the human body and invade living tissues. Screwworms (*Cochliomyia americana*) hatch from eggs laid in a mass on the edge of a wound. They should be suspected if wounds show continuous bleeding. Since the order Diptera contains so many species, it is impossible to present a simple key even for the major groups in this manual. The outline given below will serve as a convenient guide for separating some of the large groups. It should be remembered that this outline is one of convenience only, and for a more detailed classification standard texts should be consulted. Since mosquitoes constitute the most important dipterous group, their classification is treated in more detail. The technician should be able to make an examination of collected insects and to select the mosquitoes. The latter may be separated as follows:

Fragile (mosquito-like) (fig. 55 ①):

Scales on wings (fig. 52) Mosquitoes (par. 494)

No scales on wings (fig. 55 ①) Mosquito-like flies (par. 495)

Stout-bodied (housefly and horseflylike) (fig. 55 ②):

Biting flies (fig. 56 ①) Stout-bodied biting flies (par. 496)

Nonbiting flies (fig. 56 ②)
..... Filth and myiasis-producing flies (par. 497)

494. Mosquitoes

a. The family Culicidae, to which the mosquitoes belong, may be divided into two subfamilies. Of these the mosquitoes (Subfamily Culicinae) are the only ones that suck blood and are of medical interest. They are distinguished from other common mosquitoelike insects by the presence of scales on the veins and margins of the wings. (See fig. 52.)

b. Mosquitoes are classified into several genera, most of which will be omitted here since they are unimportant as disease transmitters. The forms mentioned in this manual are the malaria mosquitoes (genus *Anopheles*) and several other genera called jointly culicine mosquitoes, including the yellow-fever mosquitoes (genus *Aedes*) and the common house mosquitoes (genus *Culex*, etc.)

c. The adult anophelines (genus *Anopheles*) can sometimes be differentiated from the adult culicines (genera *Aedes*, *Culex*, etc.) by the presence of spots on the wings, these spots being almost always absent in the latter groups.

Other characters that serve to differentiate anophelines and culicines are the position of the body when at rest and the struc-



① *A. crucians*



② *A. punctipennis*



③ *A. maculipennis*



④ *A. quadrimaculatus*

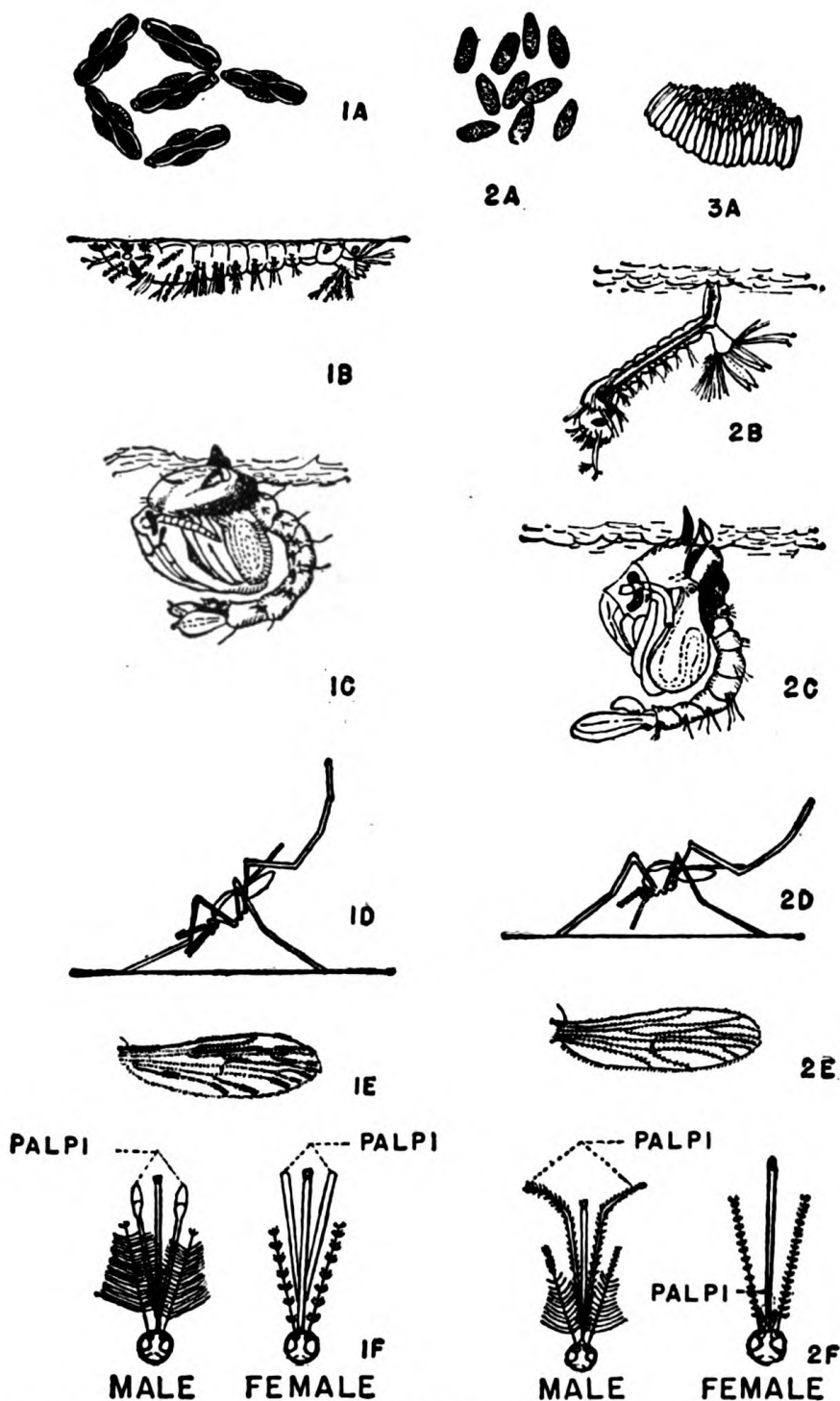


⑤ *A. pseudopunctipennis*



⑥ *A. albimanus*

Figure 52. Wings of anopheline mosquitoes showing the dark scales of the wing pattern.

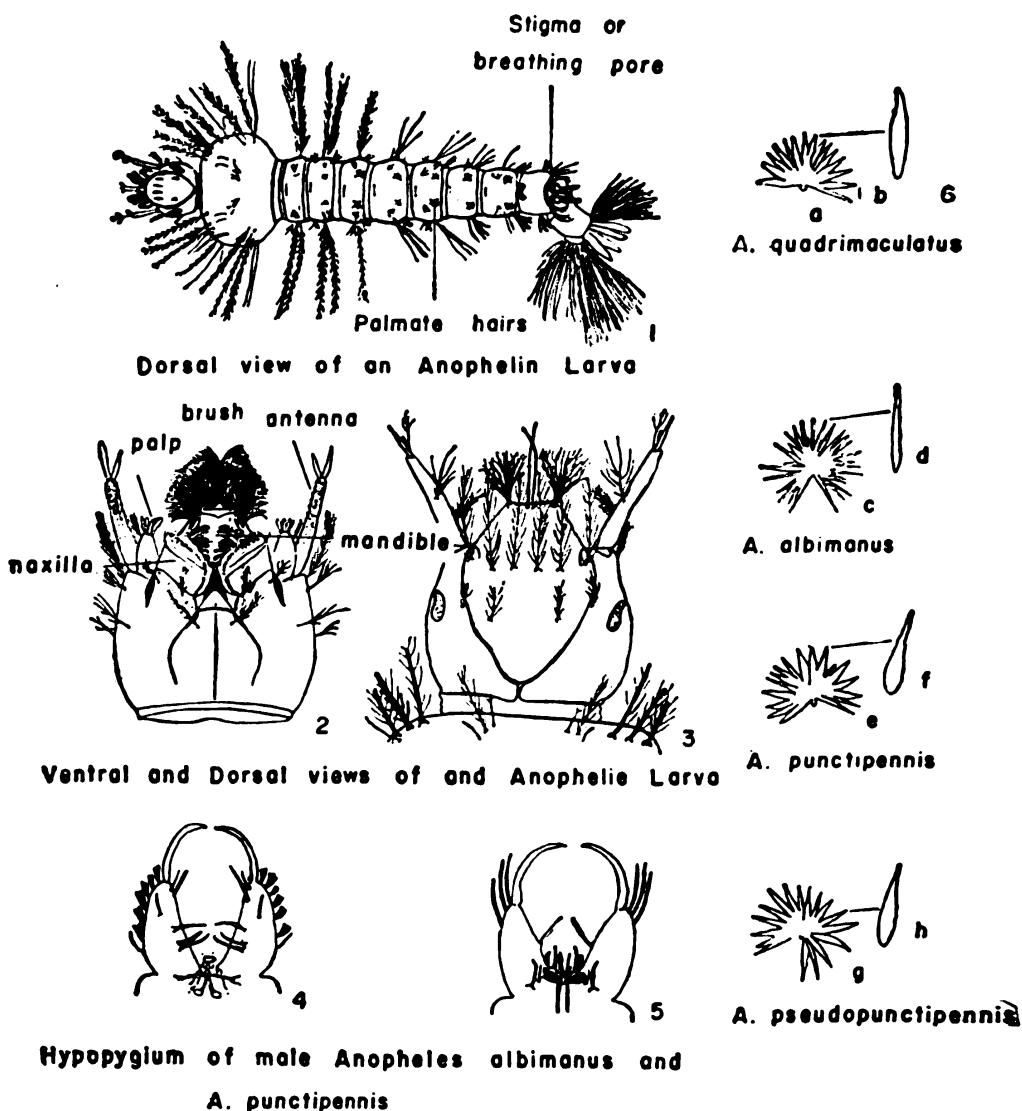


1. *Anopheles*: a. eggs; b. larva; c. pupa; d. adult; e. wing of adult; f. antennae and mouth-parts of adult male and female.
2. *Aedes*: a. eggs; b. larva; c. pupa; d. adult; e. wing of adult; f. antennae and mouth-parts of adult male and female.
3. *Culex*: a. eggs.

Figure 53. Comparison of various stages of anopheline and culicine mosquitoes.

ture of the mouth parts, especially the palpi. (See fig. 53.) Differences between eggs, larvae, and pupae of anophelines and culicines are also illustrated in figure 53.

d. To a certain extent members of the genus *Aedes* can be differentiated from other culicines by the presence of white bands on the legs, this character being generally absent in the others. The character is, however, not entirely valid and is given merely as a suggestion in tentative classification. For more accu-



(Note. 1. Dorsal view of an Anopheline larva. 2. Ventral view of the head of an Anopheline larva. 3. Dorsal view of the head of an Anopheline larva. 4. Hypopygium (male sex organ) of an adult *A. albimanus*. 5. Hypopygium of adult male *A. punctipennis*. 6. Palmate hairs (hair tufts) of various Anopheline larva; a, c, e, and g are the hair tufts; b, d, f, and h are the individual hairs.)

Figure 54. Structural characteristics of *Anopheles* mosquito, showing larva and detailed structures of adult males.

rate identification the worker is referred to standard texts and other references listed in a subsequent paragraph.

e. Malaria, an extremely frequent and sometimes fatal infection caused by various protozoan parasites (*Plasmodium*) and discussed elsewhere in this manual, is transmitted by mosquitoes of the genus *Anopheles*. Among the more important species are *Anopheles quadrimaculatus* and *A. maculipennis* in the United States and *A. albimanus*, *A. pseudopunctipennis*, and *A. darlingi* in Mexico and Central America. In the West Indies, Panama, and northern South America, *A. albimanus*, *A. pseudopunctipennis*, *A. punctimacula*, *A. aquasalis*, *A. bellator*, and *A. albitarsis* are the principal malaria-carrying species. In Europe several races of *A. maculipennis* are efficient vectors. *A. gambiae* and *A. funestus* are leading vectors in tropical Africa. In Asia *A. stephensi*, *A. superpictus*, *A. culicifacies*, *A. fluviatilis*, *A. philippinensis*, and a number of other species are proven vectors. *A. hyrcanus* and *A. maculatus* are important in Burma and Malaya. Most important in the Philippines is *A. minimus* var. *flavirostris*. The wings of several common species are illustrated in figure 52. Larval characters are shown in figures 53 and 54.

f. Yellow fever is a virus disease with a high fatality rate. In its epidemic form it usually is transmitted from person to person by *Aedes aegypti*. Yellow fever occurs in the jungles of South America and Africa, where it is transmitted by various forest mosquitoes to reservoir hosts, including monkeys. *Aedes simpsoni*, *A. leucocelaenus*, and *Haemagogus capricornii* are known vectors. Forest workers become infected and may be starting points for epidemics if they reach town where *A. aegyptii* is common.

g. Dengue (breakbone fever) is a virus disease transmitted by mosquitoes. Although it has a low fatality rate, during epidemics it causes a great deal of disability. Dengue is known to be transmitted by *Aedes aegyptii* and *A. albopictus*.

h. Filarial worm infections are also transmitted by many species of mosquitoes. The commonest of these worms are *Wuchereria bancrofti* and *W. malayi*. In these diseases, mosquitoes serve as intermediate hosts, and when they feed on a person the filarial larvae are transferred to the human host. The common house mosquito of the tropical or subtropical portions of the world, *Culex quinquefasciatus* (*C. fatigans*), is known to be the important vector in filarial infections by *W. bancrofti*.

i. Certain neurotropic viruses causing varieties of encephalitis have been shown to be transmitted by various species of *Culex* or *Aedes*. Among these diseases are St. Louis encephalitis, Japanese encephalitis, and Western and possibly Eastern equine encephalomyelitis.

j. Authoritative references to mosquitoes and their control will be found in *Keys to the Anopheline Mosquitoes of the World*, by P. F. Russell, L. E. Rozeboom, and A. Stone (American Entomological Society, The Academy of Natural Sciences, Philadelphia—1943) and in *The Mosquitoes of the Southeastern States* by W. V. King, G. H. Bradley, and T. E. McNeel, U. S. Dept. of Agriculture, Misc. Publ. No. 336, Washington, D. C.—1944.

495. Mosquito-like Flies

These are mostly small flies that may be confused with mosquitoes. They are differentiated from that group, however, by the absence of scales on the wings.

a.

- (1) A group of small, hairy flies, belonging to the family *Psychodidae*, are for the most part harmless, but the females of one widely distributed genus, *Phlebotomus*, are annoying nocturnal bloodsuckers. Some species of the group transmit diseases. *Phlebotomus* may be recognized by the position of the wings, which are held upward and outward when at rest.
- (2) Pappataci fever (sandfly fever) transmitted by *Phlebotomus* in the Mediterranean region, Balkans, Near East and India, is of military importance, since large numbers of men may be incapacitated simultaneously, although there is no mortality.
- (3) *Phlebotomus* has been shown to transmit Carrion's disease (Oroya fever or verruga peruana) in Peru and also in Ecuador and Columbia. Kala azar and oriental sore are also transmitted by these insects.

b. The blackflies (Simuliidae) include one group of medical interest, the genus *Simulium*. Certain species are very annoying and some are instrumental in the transmission of onchocerciasis in Africa, Guatemala, and southern Mexico.

c. The midges (Chironomidae) are most liable to be confused with mosquitoes. The larvae of this family are the familiar "blood worms" so often found in stagnant water. Midges of the genus *Culicoides* belonging to the related family *Ceratopogonidae* are very annoying biters. Of more importance is the fact that some members of this genus transmit certain filarial worms from man to man in tropical regions. None of these worms causes a definite disease however.

d. The crane flies (Tipulidae) are of no medical importance but are mentioned in passing. (See fig. 55①.) These large, mosquito-like forms are commonly encountered in warm weather and are

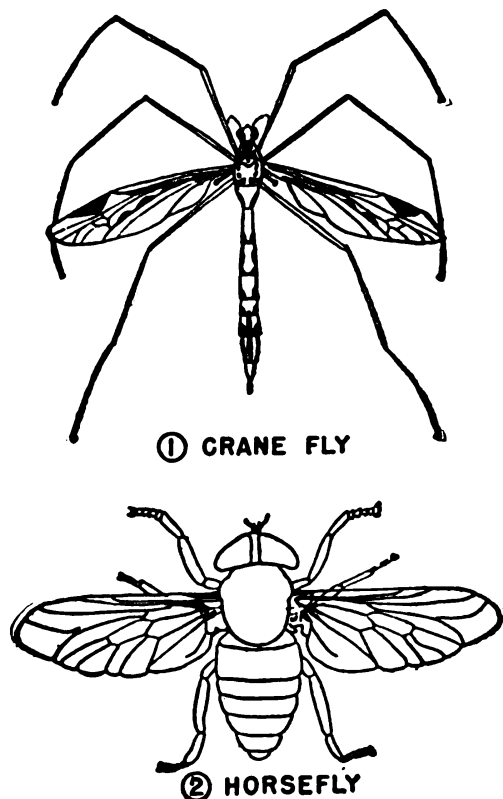
almost always thought by the layman to be large mosquitoes. They may be differentiated from mosquitoes quite easily, since they have no scales on the wings.

496. Stout-bodied Biting Flies

This group includes the horseflies, deerflies, stableflies, and tsetse flies. The species of this group are of interest because of disease transmission as well as of annoyance from their bites.

a. The horseflies (*Tabanus*) (fig. 55②) and deerflies (*Chrysops*), both of which belong to the family Tabanidae, are voracious blood-feeders. In addition to this annoying habit, which is limited to the females, species of *Chrysops* transmit a filarial worm (*Loa loa*) and tularemia. Species of *Tabanus* have been recorded as transmitting anthrax to man and tularemia to guinea pigs.

b. The biting stablefly (*Stomoxys calcitrans*) (fig. 56) of the family Muscidae, is a biting fly that closely resembles the common



① Crane fly. Long delicate legs, slender abdomen.

② Horse fly. Large compound eyes almost contiguous, characteristic Y-shaped branching radial veins.

Figure 55. Typical flies.

housefly in appearance. Occasionally before a rain it will be noticed that the flies are annoying and may even bite. When this

occurs, it is not the housefly, as is commonly believed, but the stablefly or so-called "dog-fly." It may be differentiated from the housefly by the biting mouth parts. (See fig. 56.) It has been suggested, but not proved, that the stablefly is a vector of poliomyelitis, anthrax, and tetanus. Both males and females feed on human beings.

c. The tsetse flies (*Glossina*) of the family Muscidae are of great importance in parts of Africa, where certain species transmit Gambian and Rhodesian sleeping sickness. Both males and females take blood meals. The trypanosomes causing sleeping sickness may be transmitted directly through mere mechanical transfer or after undergoing development in the tsetse fly. These flies do not lay eggs but deposit full-grown larvae.

497. Nonbiting Flies (Filth Flies and Myiasis-producing Flies) (fig. 56)

a. This group includes the houseflies, flesh flies, blue and green metallic-colored flies, and other species usually associated with decaying filth and garbage. They are of interest because they produce disease in two ways—by mechanical transmission of harmful organisms or by direct invasion of their maggots into the human body.

b. The mechanical transmission of disease is easily accomplished by the flies' habits of frequenting filth, garbage, excreta, etc. The housefly is important in the transmission of many human diseases, particularly typhoid fever, dysentery, and cholera. In addition to transmitting disease by passing from infectious material to food, it lays eggs in the infective filth, and the maggots and

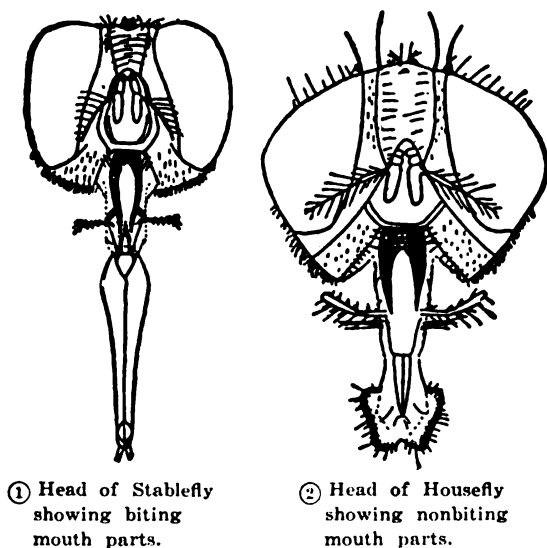
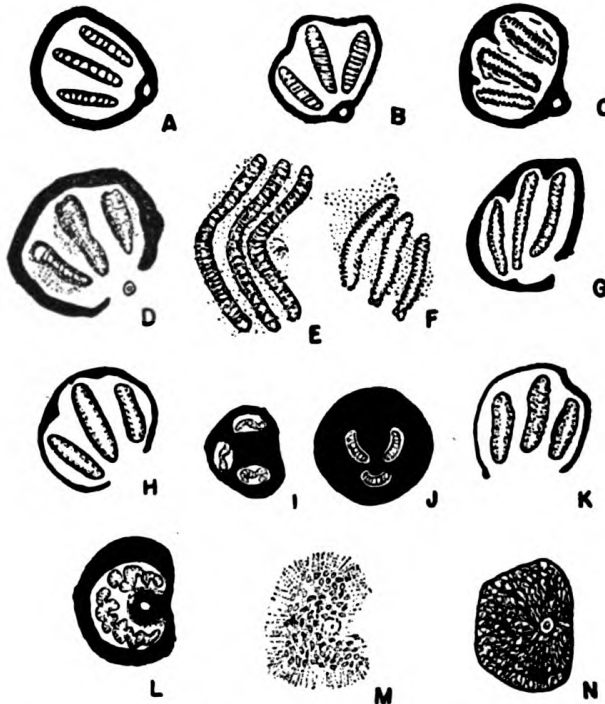


Figure 56. Fly mouth parts.

adults that follow may be infected with the pathogenic organisms.

c. The housefly (*Musca domestica*) is the most important member of this group. Others are flesh flies (*Sarcophaga*), the nonbiting stablefly (*Muscina stabulans*), the lesser housefly (*Fannia canicularis*) and the blue-bottle and green-bottle flies (*Cochliomyia*, *Lucilia*, *Phormia*, *Calliphora*, and *Chrysomya*).

d. In addition to transmitting human disease, some of the above, as well as other species, infest man directly. This is accomplished by the gravid females depositing eggs (or larvae) on wounds or open sores. The eggs hatch, and the larvae penetrate the tissues, causing myiasis. Some species lay eggs in the nostrils of sheep, goats, and deer, and occasionally infest man. The maggots developing in the nasal passages have been known to cause extensive tissue damage and even death. Intestinal and urinary myiasis are also known. Although in many cases invasion by these maggots facilitates the entrance of harmful bacteria, some fly larvae are known to keep wounds clean. Maggots living in the wounds of soldiers injured in battle have sometimes been noted to remove the debris and bone fragments and promote rapid healing by secretions from their bodies. More recently extracts of



A, Blowfly (*Calliphora*); B, Green-bottle fly (*Lucilia*); C, Blue-bottle fly (*Cynomyia*); D, Screwworm fly (*Cochliomyia*); E, Botfly (*Gasterophilus*); F, Warble fly (*Dermatobia*); G, Flesh fly (*Sarcophaga*); H, Black blowfly (*Phormia*); I, Biting stable fly (*Stomoxys*); J, Nonbiting stable fly (*Muscina*); K, Flesh fly (*Wohlfahrtia*); L, Housefly (*Musca*); M, Cattle botfly (*Hypoderma*); N, Sheep botfly (*Oestrus*).

Figure 57. Stigmal plates of fly larvae.

maggots have been used in the treatment of certain types of wounds.

e. Among the more important myiasis-producing flies are the flesh flies (*Sarcophaga* and *Wohlfahrtia*), tumbu fly (*Cordylobia anthropophaga*), screw-worm fly (*Cochliomyia*), blue-bottle fly or blowfly (*Calliphora*), green-bottle fly (*Lucilia*), housefly (*Musca*), warble-fly (*Dermatobia*), botfly (*Gastrophilus*), lesser housefly (*Fannia*), cattle grub (*Hypoderma*) and sheep bot (*Oestrus*).

f. Identification of adult flies is difficult and will not be attempted herein; reference should be made to standard texts. Fly larvae may be found in feces sent to the laboratory for examination and should not be confused with parasitic worms; often they represent contaminations of fecal material. Maggots (fig. 51) may be presented for identification from breeding areas around Army camps. These larvae, especially in the stage immediately preceding pupation, can be identified by the pattern of the stigmal plates. (See fig. 57.) These plates are a pair of tiny chitinous structures located at the hind end of the maggot. They may be removed from the maggot with a razor blade and can be identified under a microscope. In figure 57 only the left stigmal plate is illustrated. For further descriptive details, standard texts should be consulted.

498. Hemiptera (Bedbugs, Blood-sucking Conenoses, Kissing Bugs or Assassin Bugs, Stinkbugs, etc.)

Although the term "bug" is applied to all members of the class Insecta, strictly speaking only the members of the order Hemiptera are true bugs. True bugs are very common, many living in water, others abounding on plants and feeding on their juices. Some are blood suckers and are troublesome or even dangerous to man.

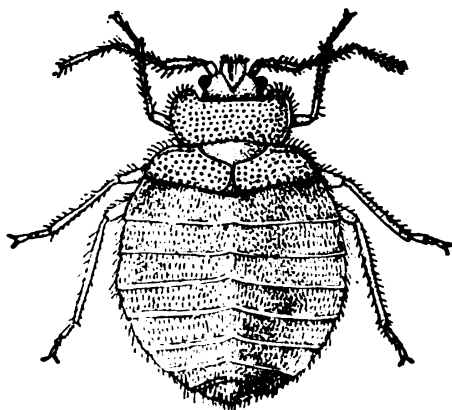


Figure 58. Bedbug.

a. The common bedbug (*Cimex lectularius*) (fig. 58) is world-wide in distribution and is a temporary parasite of man, feeding on blood and living and breeding in the cracks and crevices of beds and other furniture, and in the walls and floors of barracks, houses, etc. The adult is 4–5 millimeters long and 3 millimeters broad. In the absence of the human host, bedbugs feed on other animals. Although the bedbug has been charged with the transmission of various human diseases, there has been no definite proof of this, and the insect's only interest to the soldier is the local irritation and general annoyance that are produced by its bites.

b. Certain assassin bugs are of definite medical interest in some localities. These bugs, variously called kissing bug, blood-sucking conenoses, and assassin bugs (see fig. 59), are important in the transmission of Chagas's disease. This serious and often fatal disease, caused by a trypanosome (*Trypanosoma cruzi*), is chiefly one of childhood, but it may occur also in adults. It is found in South America, especially Brazil, where the barbeiro, *Triatoma megistus* (synonyms, *Conorhinus* and *Panstrongylus*), is the principal vector of this disease. Closely related species, some of which are infected with trypanosomes, occur in the southern United States, although as yet no human cases of Chagas's disease have been reported.

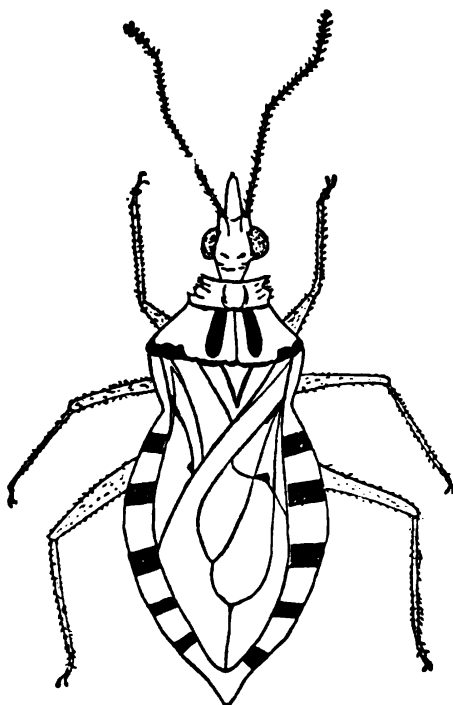
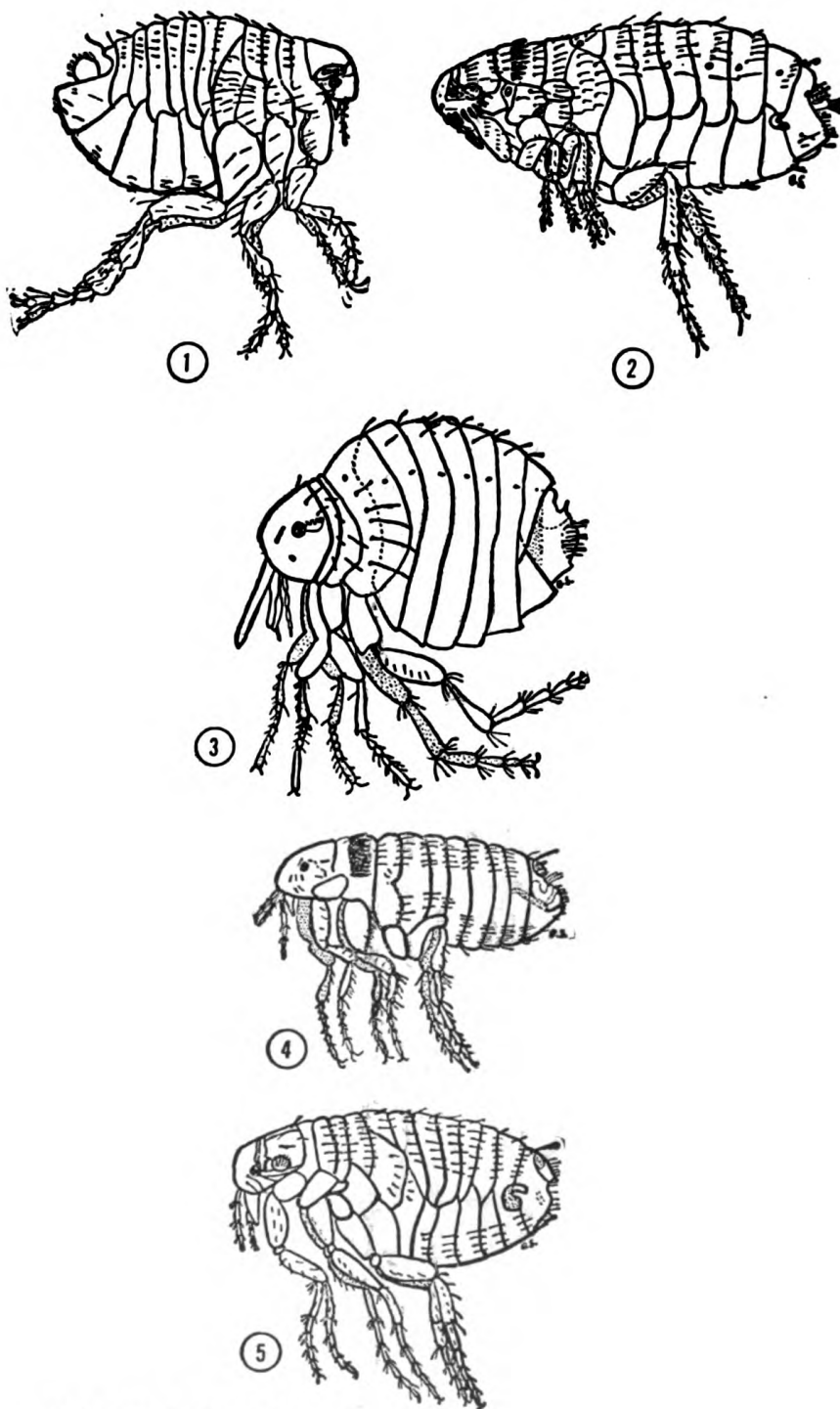


Figure 59. Blood-sucking conenose or assassin bug (Hemiptera).



- ① Human flea (*Pulex irritans*).
- ② Dog flea (*Ctenocephalides canis*).
- ③ Chicken flea (*Echidnophaga gallinacea*).
- ④ Temperate zone rat flea (*Ceratophyllus fasciatus*).
- ⑤ Tropical rat flea (*Xenopsylla cheopis*).

Note. ③ measures 1 to 1.5 mm. in length; others, about 2 mm.

Figure 60. Fleas.

499. Siphonaptera (Fleas)

The fleas are of medical importance chiefly because certain species transmit bubonic plague and endemic typhus. However, other species that may occasion considerable annoyance and irritation are commonly encountered. •

a. The human flea (*Pulex irritans*) (fig. 60①) is found wherever man lives, and is widely distributed throughout the western United States, especially in California. This flea lives in the cracks and crevices of the home and in the floors, rugs, and bedding, emerging at night to attack the host. The human flea feeds readily on dogs, rodents, and other animals as well as on man.

b. The Chigoe flea (*Tunga penetrans*) is found in tropical America, the West Indies, tropical Africa, and India. The impregnated female attaches to the skin of man, pigs, and dogs. The resultant swelling almost completely incloses the flea, which swells to the size of a pea because of the eggs developed. The eggs drop to the ground. Following egg laying the female dies. Secondary infection of the attacked sites, which in man are usually the sole of the foot, the region between the toes, and the legs, is frequent.

c. The tropical rat flea (*Xenopsylla cheopis*) (fig. 60⑤) is a most important vector of disease, particularly bubonic plague. This flea is widely distributed in tropical regions throughout the world and is found in many parts of the United States. Although primarily a parasite of the rat, during an epidemic it not only transmits plague from rat to rat, but also from rat to man, and from man to man.

d. The temperate-zone rat flea (*Nosopsyllus fasciatus*) (fig. 60④) is another species of medical importance. It, too, helps to maintain plague and endemic typhus among rats and occasionally transmits these diseases to man. It is world-wide in distribution, but for the most part is confined to the temperate zone.

e. The dog and cat fleas (*Ctenocephalides canis* and *C. felis*) (fig. 60②) are nearly cosmopolitan in distribution, particularly in temperate and warmer climates. In addition to dogs and cats, both species have a wide range of hosts, including man, rats, and various mammals. They may be found in enormous numbers in buildings frequented by dogs and cats and on adjacent premises, and are capable of transmitting dog tapeworm (*Dipulidium caninum*) to dogs and rarely to children. Infection is acquired by swallowing the flea.

f. The mouse flea (*Leptopsylla segnis*) is a common ectoparasite of mice and rats in the Eastern Hemisphere but is also widely distributed in the Americas. This species is important in transmitting plague from rat to rat.

g. The chicken flea (*Echidnophaga gallinacea*) (fig. 60③) is of interest because it is commonly encountered and may be confused with more important species. It is very similar in appearance to the Chigoe flea.

h. The most serious human disease transmitted by fleas is plague, that age-old destroyer of mankind, caused by *Pasteurella pestis*. Of all the fleas known to transmit this disease to man the tropical rat flea is by far the most important. The human flea transmits plague among small animals and in times of epidemics may transmit the disease to man.

i. Murine or endemic typhus, caused by *Rickettsia mooseri*, is also transmitted by fleas. This infection is found along the South Atlantic and Gulf Coasts and in certain other localities. It most frequently occurs in persons handling foods infested with rats. Although this disease may be transmitted to man by human body lice, it is commonly transmitted from rat to man by the tropical rat flea and the temperate-zone rat flea.

j. Some fleas, particularly the dog fleas, cat fleas, and human fleas, serve as intermediate hosts for certain helminth parasites.

Section III. COLLECTION, PREPARATION, AND SHIPMENT OF SPECIMENS

500. Collection of Lice and Mites

a. The simplest way of removing head and pubic lice from the host is by means of a fine-tooth comb. Body lice are picked from clothing or bedding with forceps. It is always best to keep the lice alive until they have digested the blood meal if they are to be preserved for identification.

b. Parasitic mites are usually collected by scraping the skin. In cases of dead animals, parts of the diseased skin should be removed and preserved.

501. Preparation of Lice and Mites for Study

a. Lice may be forwarded to specialists for identification by placing them in small vials containing 70 percent ethyl alcohol to which 1 percent glycerine has been added. Each vial should contain the name of the collector, locality, date of collection, and host.

b. Permanent microscopic mounts of mites, lice, and many other small arthropods can be made readily in chloral gum medium. Specimens that are not heavily pigmented are removed from the preservative, washed in water, and mounted directly in this medium. Specimens heavily pigmented should be boiled in a 10-percent solution of potassium hydroxide and washed in water

before mounting. Live specimens may be killed in hot water and mounted directly in the chloral gum. The slide should be labeled with the name of collector, locality, date of collection, and host.

c. The chloral gum is prepared as follows:

Gum arabic	20 gm
Distilled water	35 ml
Glycerin	12 ml
Chloral hydrate	30 gm
Glucose	3 gm

The gum arabic is dissolved in the water, and the other ingredients are added in the order given. The thick solution is strained through thick muslin for clearing.

d. Permanent balsam mounts of lice and mites can be prepared in a similar manner to the procedure described in paragraph 507 for mounting fleas.

502. Collection of Ticks

a. Hard ticks (*Ixodidae*) may be collected from their host by examination of all parts of the animal, particularly around and in the ears, back of the head, above the root of the tail and inside the flanks. Care must be taken in removing ticks in order that the capitulum (mouth parts) does not break and remain imbedded in the skin of the host. Chloroform will usually induce the ticks to withdraw the capitulum.

b. Tick specimens are sometimes collected by dragging a square piece of white cotton flannel over the vegetation. Certain ticks (*Argasidae*) are collected in the habitats of their hosts, whereas some of the *Ornithodoros* species bury themselves in the soil and can be collected by sifting soil over white paper.

503. Preparation of Ticks for Study

Tick specimens should be preserved in small vials containing 70 percent ethyl alcohol containing a small amount of glycerin. Live ticks, in vials, will ship very well if provided with a reasonable amount of moisture.

504. Collection of Mosquitoes

Mosquitoes are usually collected in a chloroform tube or cyanide jar while they are attempting to bite. The larvae are collected by dipping in favorable breeding places. In order to obtain an index of mosquito abundance at Army posts and to check on the effectiveness of control measures, a uniform system of collections should be instituted. For this purpose; it is suggested that four classes of collections be made so far as possible:

a. ADULT MOSQUITO STATION COLLECTIONS.

- (1) Anopheline mosquitoes have the habit of resting during the daytime in dark, cool shelters, usually inside or underneath buildings near the source of their blood meals, but also in other places, such as culverts, boxes, hollow trees, etc. A series of 10 to 25 of the most favorable of such shelters should be selected and given station numbers. These stations should be in the cantonment area or within $\frac{1}{4}$ mile of the barracks. The station may consist of only a portion of a room or building if other parts are not readily accessible for inspection or if it is found that the anophelines are mostly congregated in one large place, as is frequently the case. The inspections should be made weekly, or oftener if indicated.
- (2) The collections in these shelters are made with the aid of a flashlight, and the mosquitoes are captured with a chloroform killing bottle or an aspirator tube. Resting mosquitoes will usually fall into the open mouth of a killing tube brought up slowly from below.
- (3) For information concerning the abundance of mosquitoes outside of the cantonment zone a second series of stations can be selected, if desired, but the records for this series should be kept separate.
- (4) Wooden boxes, with an end opening of 12 to 18 inches square and painted brick red inside, may be used in places where other shelters are not available. They are of special advantage near breeding places where they serve as a check on the effectiveness of oiling. They should be placed in the shade, on or within three feet of the ground.

b. LIGHT TRAP COLLECTIONS.

- (1) The New Jersey type of light trap, equipped with a light bulb and a suction fan, is useful to sample the mosquito population of an area and to record fluctuations in abundance. These traps may be improvised, if material is available.
- (2) Two to four traps, depending somewhat on the size of the cantonment area, should be placed at favorable locations for sampling the mosquitoes that actually invade the camp. The traps should be hung on the arm of a standard or on a tree with the light at least 6 feet from the ground. They are more effective if set up where the light can be seen from all directions and should not be placed near a street light or a building in which a bright light is kept burning.

- (3) For uniformity, a 25-watt white bulb should be used and the traps should be allowed to run all night. Operation of the traps three to five times per week has been found to give an adequate record of mosquito abundance.
 - (4) Sodium or potassium cyanide makes the best killing jars for these traps, but paradichlorobenzene (Dichlorocide) may be used if necessary. On rainy nights some moisture may collect in the jars and damage the specimens for identification. To overcome this to some extent it is advisable to use a small paper cup (a paraffin drinking cup or an ice cream container) punctured with small nail holes and of the proper size to fit the mouth of the mason jar. A cylinder of fine-meshed wire may also be used. These hold the specimens but permit the water to pass through.
 - (5) Some species of mosquitoes are not attracted by light to the same degree as others and this appears to be the case with *Anopheles quadrimaculatus* and *Culex quinquefasciatus*. This fact should be kept in mind in attempting to interpret the relative abundance of these species in comparison with others. Nevertheless, the trap collections will furnish information even for these two species regarding increases or decreases that may occur from week to week.
- c. BITING COLLECTIONS.
- (1) Records of the number of mosquitoes attempting to feed give direct information on the kinds that are causing annoyance and their relative abundance. These records should be made after dark and can be obtained either by two persons, one collecting from the other, or by one person collecting alone. In the latter case, it is best for the collector to be seated on a box or stool with the trouser legs rolled above the knees. The specimens are captured with a chloroform tube or aspirator as they alight or begin to puncture. A flashlight is used but the amount of light should be reduced by removing the lens or covering it with semitransparent paper. Too bright a light may frighten many specimens away. The collecting at one place should be done for a set period, usually 30 minutes if the numbers are small.
 - (2) Collections should be made weekly at several points in the camp if possible. These records are of particular value in furnishing information or answering criticism concerning the actual degree of annoyance.

d. LARVAL COLLECTIONS.

- (1) A system of larval collections or inspections should be instituted to provide exact information on the areas producing species of medical importance, the need for larval control, the effectiveness of the larvicidal program, and the changes in breeding conditions that may occur.
- (2) As a preliminary to this the camp should be divided into a few zones or areas, based if possible on natural divisions, and then subdivided into convenient units designated by letters or numbers. Within the different units the ponds, if numerous, can be numbered and also different sections of the streams. The purpose is to have a convenient means of designating each potential breeding place to record the larval inspections and to give specific locations to the larviciding crews. Each pond or section of streams or area of small pools is referred to as a larval station or collecting area. A general description of the station should be written up at the beginning of the season showing the type, area, average and maximum depths, amount and principal kinds of vegetation, etc.
- (3) The larval collections are usually made with a white-enamel dipper having a hollow handle into which a round stick or piece of cane can be inserted. Shallow pans are preferred by some collectors. If a graniteware or tin dipper only is available it is advisable to paint the inside white as the larvae are more easily seen against a white background.
- (4) In order to provide records regarding the comparative abundance of larvae at different times or in different areas the dipping should be done in a systematic and uniform manner. For *Anopheles*, the dips are made by skimming through the surface of the water in places where aquatic vegetation or floating debris offer favorable conditions for the larvae. Since it is desired to obtain a fair average of the larvae present, the dips should be well scattered over the station at intervals of 10 to 15 feet. The tendency to make most of the dips in a few of the most favorable spots should be avoided. The dips are usually taken in multiples of 10 and the number taken at each station is recorded on the notes. The average number of larvae present are given as the number per 10 dips.
- (5) The larvae are pipetted from the dipper into a collecting bottle, preferably a wide-mouth bottle of 60 ml to 120 ml capacity provided with a cork stopper. It should be labeled with the station number and date. In warm

weather the bottle should be filled not more than half full of water and the cork loosened occasionally to provide air. The bottles should never be set in the sun or left in a closed car in the sun for any length of time. An open glass tube, run through the cork nearly to the water line aids greatly in maintaining a constant pressure and providing air without danger of leakage. A wide-mouthed pipette with a rubber nipple is used to remove the larvae. If an ordinary medicine dropper is the only type available the small end can be filed off and the sharp edges annealed in a flame. For killing and preserving of larvae see paragraph 505.

- (6) Inspections should be made weekly in stations known to be favorable for breeding or those that have been put on a weekly larviciding schedule. Other areas that have proved to be of little importance as breeding places should be inspected at least once a month.
- (7) Temporary pools that produce *Aedes* and *Psorophora* should be inspected after each rain.
- (8) The mosquito material should be identified so far as facilities and experience permit by the station personnel and then mailed in suitable containers to a central laboratory or a specialist for confirmation.

505. Preparation of Mosquito Specimens for Study

a. Mosquito adults are very delicate and must be handled carefully to avoid loss of scales or appendages. Moisture is also damaging, since the scales easily lose their natural color. Condensation of moisture on the inside of a chloroform tube occurs very quickly when it is left in the sun or a heated place. Furthermore, specimens are usually ruined when left too long in a relaxing jar for softening. Mosquitoes captured in a killing tube should be left only long enough to be certain that they will not recover, and then removed to a pillbox.

b. Specimens kept in the office in small boxes, even in apparently tight pillboxes, are frequently destroyed by ants or psocids. The boxes should be stored in a larger box or jar containing naphthalene or paradichlorobenzene. For shipment or transportation specimens should be packed as soon as possible after killing and placed in pillboxes or similar containers preferably between layers of cellu-cotton, lens paper, cleaning or soft toilet tissue, if available. Ordinary toilet tissue tends to rub off scales but may be used if it is the only available material. If cotton is used as a filler it should not be in contact with specimens. Packing must be

sufficient to prevent movement. A few fine flakes of naphthalene should be added if possible to protect the mosquitoes against insects, molds, etc., but this material should not be in direct contact with the specimens.

c. Each pillbox should contain material from only a single collection and should be labeled with the name of the post, the date of collection, and either the station number or individual collection number. They should be packed in a mailing tube or a box for shipment.

d. Adult specimens that have been mounted on pins or paper points may be shipped in a suitable pinning box. Pins should be set in firmly by use of pinning forceps.

e. Mosquito larvae may be reared through to the adult stage, or fourth instar larvae may be preserved for identification in small vials containing formalin or alcohol (10 percent commercial formalin or 70 percent ethyl alcohol).

f. Larvae are best killed by immersion in hot but not boiling water. A temperature of 50°–65° C. is recommended. Several changes of alcohol, beginning at 30 percent and running up to 70 will insure a minimum of distortion. For prolonged storage, however, formalin is usually preferred. Each vial must be labeled with similar information to that indicated above for the adults.

506. Collection of Fleas

a. Fleas may be collected from infested persons or animals by picking. When fleas are desired from small wild animals the hosts should be captured by traps that take them alive, since fleas soon leave a dead animal. The host should be anaesthetized or killed, using ether or chloroform, and its fleas and other ectoparasites dislodged with a fine-toothed comb or with forceps. With rats and other rodents it is usually easier to obtain the animals in traps that kill them. Large numbers of fleas are usually lost when this form of trapping is used but enough may remain on the animals to make it worth while. Some animals may be clubbed or shot in order to obtain specimens of fleas. Fleas may also be collected from the nests and burrows of the host animal.

b. The dead animal should be placed immediately into a paper bag where fleas, mites, lice, and ticks can be collected. It is advisable to stupefy the fleas with an insect powder before combing them out onto a white paper. Fleas can also be collected from recently killed rats by combing them out over water. The fleas jump from the dead host into the water from which they can easily be collected.

507. Preparation of Fleas for Study

a. Fleas may be preserved in 70 percent ethyl alcohol containing 1 percent glycerin or they may be prepared for study by mounting them in chloral gum or canada balsam on glass slides. The following procedure is recommended for preparing permanent microscopic mounts of flea specimens:

- (1) Drop the living flea or one that has been killed in alcohol into 10 percent potassium hydroxide and allow it to remain for 1 or 2 days.
- (2) Transfer specimen to water containing a few drops of hydrochloric acid and allow it to stand for 30 minutes.
- (3) Transfer specimen to 50 percent ethyl alcohol for 30 minutes.
- (4) Transfer specimen to 95 percent ethyl alcohol for 30 minutes.
- (5) Clear in creosote for 1 hour.
- (6) Place specimen on a clean glass slide and mount in canada balsam.

b. Microscopic mounts of flea specimens can also be made in chloral gum medium, as described in paragraph 501.

508. Collection and Shipment of other Insects of Medical Importance

a. Houseflies and blowflies may be collected in flytraps of the type for housefly control on military reservations. Other insects of medical importance may be collected in specifically designed traps, with insect nets or by hand picking. These insects can be killed in an ordinary chloroform killing tube generally used for collecting mosquitoes or in a cyanide bottle of the type used by entomologists for killing larger insects. Insects can also be killed by placing a piece of cotton moistened with chloroform with the insect in a tight-closing jar.

b. These insects should be packed in pillboxes in the same manner that adult mosquito specimens are packed for shipment. Each box containing an insect collection should have the name of the collector, date, and locality of the collection written plainly upon it.

CHAPTER 16

PATHOLOGIC METHODS

Section I. GROSS PATHOLOGY METHODS

509. Morgue

a. GENERAL. The morgue should have good light (natural and artificial) and ventilation, as well as running water and gas. A floor drain and a water tap to which a short hose is attached are desirable. A cabinet should be provided for the instruments and these should be cleansed in water and immersed in a 5-percent solution of Cresol Comp. *U.S.P.* (cresol, saponated solution) or equivalent solution for not less than 20 minutes, then rinsed in water, thoroughly washed, completely dried and carefully placed in the cabinet after each autopsy. If an autopsy table is not supplied, one can be improvised by covering a wooden table with galvanized iron, sheet lead or other water proof material, allowing a gentle slope to the foot of the table and a drain with a pipe fixture to open over the floor drain. The table should be thoroughly scrubbed with the 5-percent cresol solution after each autopsy. Rubber gloves should be used by all engaged in performing autopsies or handling the organs. If not available, the hands must be kept wet continuously. The gloves should be washed while on the hands with soap and water, then reversed as they are removed and the insides similarly cleaned. They should then be thoroughly washed in soap and water, dried and covered with talcum inside and out. Special precautions should be taken when performing autopsies on infectious cases.

b. COLLECTION OF SPECIMENS. Specimens removed during the autopsy for histologic examination are placed immediately in fixing solution, avoiding contact with water. Gross specimens that are to be preserved should not be allowed to dry, but placed in a preserving solution as early as possible.

510. Care and Restoration of Body

At the conclusion of the autopsy all excess fluid should be removed from the cavities, the rectal, vaginal, and urethral openings closed; the organs not retained for further study and the sternum replaced, the body cavities stuffed with oakum, old rags, or newspapers, and the incision sewed, using the "baseball" stitch. Begin at the upper end of the incision, sew from within out, taking

liberal bits of skin and muscle, keep the string taut, and use uniform stitches about 1 cm apart. The body is then thoroughly washed, taking care to remove all blood stains especially from the hair, face, and hands. If the head has been opened, the base of the skull is filled with plaster of paris, the skull cap replaced and the scalp sutured as above. The brain is not replaced in the skull but with the other organs in the body cavity. If the spinal canal has been opened, it is stuffed with cotton or oakum over which the neural arches are replaced and the incision then sewed as above. It is impossible to exaggerate the importance of proper care and restoration of the body. The cutaneous surface should not be disfigured more than necessary, and the incisions should be hidden by the clothing.

511. Embalming

a. PREPARATION OF BODY. The embalming of the head is readily done by the undertaker through the carotid arteries when the chest is open, but in his absence may be done by the operator. If shaving is necessary it must be done before the face is embalmed. The undertaker's pressure bottle with several tubes armed with long metal cannulas, which are tied into the carotids and subclavian arteries, is most convenient. Pressure is obtained with a pump. The nozzle is tied into the upper thoracic aorta. The open end of the aorta as well as any leaking arteries' (internal mammaries) must be closed with clamps or tied. It is advisable to inject the embalming fluid into the carotid arteries before opening the head, except when bacteriologic studies are required.

b. TECHNIC. Undertakers' embalming fluid or a 10-percent solution of formalin in water, to which a few drops of eosin solution are added to give it the faintest possible tinge of pink, may be used. As the fluid is pumped into the arteries and begins to drive blood before it out of the veins, the face and ears must be massaged and moulded with a gauze sponge into a natural pose, with eyes and lips closed. The hands should also be massaged until white. When the tissue becomes blanched and firm, the process is complete. The same procedure is applied to the legs, the fluid being injected through the femoral arteries. Some formalin should be allowed to stand for a time in the body cavity. It is well to soak the organs in a 10-percent formalin solution for several hours before replacing them in the body, making incisions in the solid organs and numerous punctures in the gastrointestinal tract. Undertaker's hardening compound, oakum, or cotton should be spread over the organs after they have been replaced.

512. Fixation of Tissue

Pieces of tissue not more than 0.5 cm in thickness will be selected from representative parts and fixed in approximately twenty times their volume of 10-percent formalin. The formalin solution will be changed the following day and again immediately before packing for mailing. Such tissue is designated "wet tissue."

513. Preparation for Mailing Blocks of Tissue

When mailing, use a wide-mouth screw-cap bottle of 60 ml or 120 ml capacity depending upon the amount of tissue. The half-pint fruit jar is suitable for autopsy tissues or large surgical specimens and may be mailed in the double mailing case. The double mailing case is suitable for two 60 ml wide-mouth bottles or one 120 ml bottle. Shipments exceeding 4 pounds in weight may be made by express or if under 70 pounds and less than 100 inches in length and girth combined, may be mailed at parcel post rates, but *not* franked.

514. Gross Specimens

a. GENERAL. If preservation of color is not essential, as when an organ is to be sent to the Armed Forces Institute of Pathology for examination, fix in abundant 10-percent formalin, taking care that all parts are in contact with the solution. Bulky specimens, such as a liver, should be sliced into thick slabs. After fixation, they may be shipped in a minimum amount of solution, using any watertight container available.

b. PRESERVATION OF COLOR.

(1) Fixation.

- (a)* To preserve color in gross specimens they should be quickly washed in water to remove any excess of blood and placed in Kaiserling's solution No. 1. It is necessary to arrange the specimens in this solution as they are to appear when finally mounted, as they become fixed when placed in the solution and it is difficult to alter them afterward. The length of time in the No. 1 solution varies from 1 to 7 days, depending on the size of the specimen.
- (b)* If the specimens are very large, it is advisable to inject fixative into the blood vessels, or to impregnate the tissue by means of a syringe and needle. The specimen should be supported by cotton or suspended by strings so that it will not be in contact

with the container. Specimens should be kept in an opaque covered container protected from light.

(2) *Care after fixation.* After fixation the specimen is drained and blotted, then placed in 95-percent alcohol (Kaiserling's solution No. 2). When the maximum color has returned, which it will do in a few minutes to several hours, the specimen is removed from the alcohol, thoroughly washed and then preserved in Kaiserling's solution No. 3. It is necessary to watch the development of the color, for after it has reached a certain point it will begin to fade and cannot be restored. Furthermore, overfixation in solution No. 1 should be avoided; therefore, if the specimen is to be shipped some distance, it should be run through the alcohol and forwarded in solution No. 3. It is possible to develop some color in formalin-fixed tissue, but this method is not satisfactory.

(3) *Preparation of solutions.*

(a) *Kaiserling's solution No. 1.*

Formalin	400 ml
Water	2,000 ml
Potassium nitrate	25 gm
Potassium acetate	50 gm

(b) *Kaiserling's solution No. 2.*

Alcohol	95 per cent
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(c) *Kaiserling's solution No. 3*

Potassium acetate	200 gm
Glycerol	400 ml
Sodium arsenate	100 gm
Water	2,000 ml

Note. If sodium arsenate is not available some crystals of thymol, menthol, or sodium salicylate may be used instead, but the arsenate is preferable as a fungus deterrent.

515. Preparation

a. The preparation of museum specimens must be left largely to the ingenuity of the operator and only a few general principles can be given. The surface to be displayed should represent as large a section as possible of the whole organ, and both the exterior and interior of the organ should be shown. In the case of solid organs, such as the liver, a thick slice (3-5 cm) should be preserved, since it is impossible to fix a whole liver properly. The thickness of the slab should allow for the removal of a thin layer at a later date to freshen the surface. This is particularly true of the lung, in which case one-half or even the whole organ

may be preserved. Nothing solid should be allowed to touch the surface of the fresh tissue until it is fixed and hardened.

b. It is injurious to pack cotton firmly into a cavity, since after fixation the lining of the cavity will appear merely as a mold of the cotton. If a hollow organ must be held open, it is best to distend it with fixing fluid for a day or two before cutting into it. If this is no longer possible, and it must be propped open with cotton, this should at least be inserted very loosely.

c. The heart, after being opened, should be stretched on an improvised frame in such a way as to display to advantage the chief lesion, or it may be clamped together and held in its original form by a few temporary stitches during fixation.

d. The stomach or portions of the intestines can be filled with Kaiserling's solution No. 1 or 10-percent formalin and ligated at the ends until hardened, after which they can be bisected longitudinally. Otherwise they may be opened, stretched on a board with thread so that the mucosa is exposed and immersed in the fixing fluid.

e. One-half of the kidney, cleanly cut, forms a satisfactory specimen.

516. Specimens for Toxicological Chemistry

a. GENERAL. Medicolegal requirements govern the preservation and shipment of specimens for toxicological chemical analyses. For valid medicolegal interpretation of toxicological results there must be unbroken continuity of possession of the specimens. In part, continuity of possession means that specimens must be unchanged by external agency between the four primary places of investigation of toxicological cases: scene of the incident, autopsy room or clinical examination room, toxicological laboratory, and the court. It also means that any change in the specimens at any place of investigation must be limited to acts necessary to proper prosecution of the investigation, and must be fully recorded and documented. For these reasons, specimens must be protected against chemical and mechanical change, and in ways that are different from the preservation of specimens for pathological examination.

b. PROTECTION AGAINST CHEMICAL CHANGE.

- (1) NO CHEMICAL PRESERVATIVE should be used; for every chemical added to a specimen will interfere with one or more chemical analyses. Instead, refrigeration and rapid transportation should be arranged for shipment of specimens. Either solid carbon dioxide or ice make suitable refrigerants, provided that arrange-

PROTECTION AGAINST CHEMICAL CHANGE

TISSUE:

1. Chemically clean, glass-stoppered containers. (spring-clamped Mason jars)
2. When distance and climatic conditions permit, or when alcohol is suspected poison, use NO PRESERVATIVE, but instead ship in a refrigerated condition via air mail. Solid carbon dioxide or ice can be used as refrigerants. Arrange with transportation authorities to keep specimens cold until destination is reached.
3. Otherwise than in 2, use only 95 % ethyl alcohol as preservative, and submit some of it for analysis.

FLUIDS: 1. As for tissue (Reagent bottles).

2. As for tissue.
3. Toluene, unless it is the suspected poison, in which case use thymol.

PROTECTION AGAINST MECHANICAL CHANGE

1. Clean white cloth, or durable paper, is used as a hood over the stopper and encloses the container. The hood is tied with string and sealed, at top, bottom, free edge and knot, so that removal of the hood will break the seal.
2. Make a distinctive impression on the seal.
3. For packing use cotton, paper, excelsior, and pack snugly.
4. Ship fluids in containers in wooden boxes.
5. Ship fluids in containers in cylindrical, screw-capped cartons.
6. Each specimen should have a separate container with identifying label.
7. Labels on outer wrappings are of 3 kinds:
 - a. Address - To and From.
 - b. Toxicology specimens.
 - c. Handle with care.

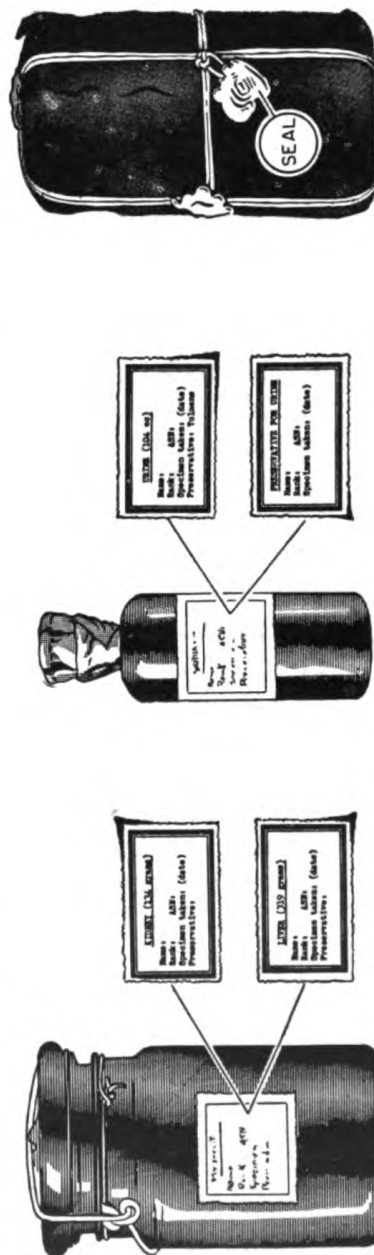


Figure 61. Shipping containers for toxicological specimens.

ments are made with transportation authorities for further refrigeration if the time of transit exceeds 24 hours. Air transport is preferable to other modes of transportation. When circumstances forbid the use of refrigerants and air transport, and when alcohol poisoning has been proved not to be a factor, by analysis of blood at the installation from which shipment is to be made, 95-percent ethyl alcohol may be used as a preservative. In this case, a sample of alcohol used for this purpose must accompany the preserved specimen. Body fluids and other liquid specimens are handled in the same way as tissues, except that thymol or toluene is substituted for ethyl alcohol as a preservative, and except that under no circumstances should a preservative be added to stomach contents.

- (2) Each specimen should be inclosed in a chemically clean container; that is, one which has been cleaned by means of dichromate-sulfuric acid mixture and has been washed by several fillings (not rinsings) with distilled water. Then the container can be drained and dried in air (not mechanically dried as by a towel), before admitting the specimen to it. The container must be glass-stoppered, of the kinds shown in figure 61, because both rubber and cork are more absorbent for certain chemicals and are more likely to be dislodged in shipment. Each container must be clearly labeled, either type-written or printed, and must show the identity of the specimen, its weight or volume, the name, rank, serial number of the subject of the investigation, the date when the specimen was obtained, the presence of alcohol, if it has been used as preservative, and the volume used, and should be clearly initialed by the responsible officer.

c. PROTECTION AGAINST MECHANICAL CHANGE. This protection includes spring clamps, as on Mason or fruit jars, and adhesive tape applied to glass stoppers when the containers are not supplied with clamps. The tape can be fixed firmly to the neck of the bottle by means of string. Each specimen container is inclosed in a clean white cloth, or durable paper, and is tied with string and sealed with wax at the top, bottom, free edge, and knot as shown in figure 61. Identification of the specimen by means of a distinctive impression in the wax, as by a signet ring, is necessary for medicolegal purposes, to indicate that the specimen has not been tampered with. The containers are packed snugly, using excelsior, cotton, or paper. In the case of large con-

tainers, the packing case is a wooden box; for smaller containers, cylindrical, screw-capped cartons may be used. Each shipping package, whether wooden box or carton, requires three labels, one to show that the contents are for toxicological examination, another directing that the package be handled with care, and the third giving the addresses of the sender and the destination. Where possible, the specimens should be sent by registered mail.

Section II. HISTOLOGICAL TECHNIC

517. Formulas

a. MAYER'S ALBUMIN.

White of egg	50 ml
Glycerol	50 ml
Sodium salicylate	1 gm

Shake well together and filter into clean bottle.

b. STARCH PASTE. Where fresh egg is not available, the following starch mixture is used. Add 1 gm of powdered corn starch to 10 ml of cold water, mix thoroughly and pour the solution into 20 ml of boiling water, then add 2 drops of 10 percent hydrochloric acid. Boil the suspension for 5 minutes, stirring constantly. A small crystal of thymol should be added after the paste has cooled. The mixture should be free from lumps when complete.

c. ACID-ALCOHOL. One percent of hydrochloric acid in 70 percent alcohol.

d. GLYCEROGEL MOUNTING METHOD FOR FROZEN SECTIONS (used when tissue is mounted directly from water).

Glycerol	20 ml
Granulated gelatins	3 gm
Chrome alum (chromium potassium sulfate)	0.2 gm
Distilled water	80 ml

Dissolve separately the chrome alum in 30 ml of water by aid of heat, and the gelatine in the remaining 50 ml of water. Combine the 20 ml of glycerol and gelatine solution while the latter is still warm. While stirring this mixture, add the 30 ml of warm chrome alum solution. When thoroughly mixed, filter and add a crystal of camphor or thymol as a preservative. If bubbles are present, put it in an oven at 37° C. until it flows freely again. Keep the bottle well stoppered to prevent evaporation. During cold weather keep it in the oven at 37° C.

e. EOSIN.

Eosin Y	0.5 gm
Alcohol	25 ml
Distilled water	75 ml

This solution will keep indefinitely.

f. HARRIS' HEMATOXYLIN.

Hematoxylin	1 gm
Alcohol	10 ml
Dissolve dye in alcohol	
Potassium alum (aluminum potassium sulfate)	20 gm
Distilled water	200 ml

For use, 8 ml glacial acetic acid is added to the mixture to increase the precision of nuclear staining.

The alum is dissolved in the water with the aid of heat, and then the alcoholic solution of the dye added. The mixture is brought to a boil rapidly and then 0.5 gm of mercuric oxide (red oxide) is added. The solution at once assumes a dark purple color, and as soon as this occurs it is cooled by plunging the flask into cold water. Potassium permanganate (0.177 gm per gram of hematoxylin) may be added in the cold if mercuric oxide is not available.

g. WEIGERT'S IRON CHLORIDE HEMATOXYLIN.

Solution A	Hematoxylin	1 gm
	Alcohol (95 percent)	100 ml
Solution B	Ferric chloride, 50-percent solution	4 ml
	Concentrated hydrochloric acid	1 ml
	Distilled water	95 ml

For use, mix equal volumes of A and B. A blue black color develops. It is best prepared fresh, although the stain will keep several days and may be used as long as it is blue to violet in color.

h. ZIEHL'S CARBOL-FUCHSIN.

Basic fuchsin	0.3 gm
Phenol	5 gm
Alcohol (95 percent)	10 ml
Distilled water	95 ml

i. OIL RED O STAIN (if oil red O is not available, Sudan III or Sudan IV may be substituted).

Oil red O	1 gm
Acetone	50 ml
Alcohol (70 percent)	50 ml

518. Frozen Section Method

It is possible with this method to prepare a slide for examination in a few minutes so that it is particularly applicable to "operating-room diagnosis." It is also the method of choice when it is desired to stain for fat.

a. MATERIALS.

Automatic freezing microtome, with a sharp knife.

Tank of CO₂ and connection; the tank to be mounted inverted.

A shallow dish filled with water.

A mounted needle, a glass rod drawn to dull point or a pair of fine, smooth-pointed forceps.

Two Pyrex test tubes.

Bottle of 10 percent formalin.
Bottle of 1 percent ammonium water.
Bunsen burner or alcohol lamp.
Glass slides and cover glasses.
Seven small glass dishes.
Harris' hematoxylin.
Eosin.
Alcohol (95 percent).
Alcohol (absolute).
Carbol-xylene (xylene saturated with phenol).
Canada balsam or Clarite.
Blotting paper.

b. TECHNIC.

- (1) If the tissue has not already been fixed, a block about 0.5 cm thick is boiled for 1 minute in 10 percent formalin in a test tube and rinsed in tap water. It is placed on the freezing stage of the microtome with a few drops of water and frozen, pressing it gently with the finger during this process. Do not freeze too hard; cut at 13 to 16 microns, remove the sections from the knife with the finger, and float them in water.
- (2) Select full sections and transfer in turn to the following, which are in small shallow glass dishes.
 - Stain in Harris' hematoxylin 30 to 60 seconds.
 - Rinse in 1 percent ammonia water until blue.
 - Rinse in tap water.
 - Stain in Eosin 5 to 15 seconds.
 - Rinse in absolute alcohol 5 seconds.
 - Rinse in 95-percent alcohol.
 - Place in carbol-xylene 5 seconds.
 - Mount on slide in Canada balsam, or Clarite.
- (3) Instead of transferring sections to staining dishes, they may be mounted on glass slides that have been smeared with egg albumin-glycerol, blotted, dried and then flooded in turn from dropping bottles containing 95 percent and absolute alcohol, followed by an extremely thin solution of celloidin. The slide should be tipped to permit the celloidin's forming a very thin film over the section. The process is then reversed, 95 percent alcohol being dropped on the mounted section. It may then be stained in the usual manner and handled exactly like the paraffin section. This method is not applicable for fat stains.

519. Routine Paraffin Embedding Method

a. MATERIALS. The following are required in addition to those enumerated in paragraph 518.

Oven with automatic control for keeping it at 56° C.

Table LXII. *Tissue Fixatives*

(Blocks of tissue should be not over 5 millimeters in thickness and should be fixed in 20 volumes of fluid)

Fixative	Use	Formula	Fixation time	Aftertreatment
Formalin alcohol.....	For rapid fixation..	Neutral formalin (40 percent)..... 10 ml Alcohol (40 percent)..... 90 ml	12 to 24 hours.....	Transfer to 80 percent alcohol.
Absolute alcohol.....	For Nissl bodies.....	Alcohol (absolute).....	12 to 24 hours (renew after 3 or 4 hours).	Transfer to 80 percent alcohol.
10 percent formalin.....	For routine use.....	Neutral formaldehyde solution (40 percent)..... 10 parts Water..... 90 parts Calcium carbonate (to neutralize).	24 hours.....	Transfer to fresh 10 percent formalin.
Zenker (acetic).....	For use with aniline dyes.	Potassium dichromate..... 2.5 gm Mercuric chloride*..... 5-8 gm Distilled water..... 100 ml Acetic acid (glacial), just before use (optional)..... 5 ml	24 hours.....	Wash 12 to 24 hours in running water; transfer to 80 percent alcohol.
Bouin.....	For skin sections.....	Picric acid (saturated aqueous solution, 1.22 percent)..... 75 ml Neutral formalin (40 percent)..... 25 ml Acetic acid (glacial)..... 5 ml	18 to 24 hours.....	Remove picric acid from section with 95 percent alcohol; transfer to 80 percent alcohol.
Regaud.....	For demonstration of micro-organisms in tissues.	Potassium dichromate (3 percent)..... Neutral formalin (40 percent)..... 80 ml Solution must be freshly made.	24 to 48 hours (renew after 24 hours).	Wash 24 hours in running water; transfer to 80 percent alcohol.

* If mercuric chloride is not obtainable, it may be replaced by 2.5 gm of zinc chloride.

Paraffin, refined (melting point 52° to 56° C.).

Containers for paraffin in oven: beakers or casseroles and Stender dishes.

Chloroform.

Acid-alcohol.

Xylene.

Paper or metal forms for molding blocks.

Two basins or photographic developing trays.

Ice.

Rotary microtome.

Sharp knife.

Hone and strop.

Camel's-hair brushes (one pointed and one 2.5 to 3.7 cm wide).

b. **FIXATION.** Specimens of tissue which are to be examined microscopically should be fixed as quickly as possible after surgical removal or after death of the patient. A 10-percent solution of formalin is the most convenient and generally practicable fixative. For finer cellular studies and some special stains, it is necessary to fix in one of the chromate solutions, of which Zenker's is the most popular. If it is desired to stain for glycogen, aqueous fixatives must be avoided as glycogen is soluble in water, and absolute alcohol must be used. Blocks of tissue should be about 0.5 cm in thickness and they should be placed in an excess of the fixative agent to insure thorough impregnation. If formalin is used, the tissue may be kept in it indefinitely. If fixation is in Zenker's, the blocks are to remain in it but 24 hours, then washed in running water for 24 hours and preserved in 80 percent alcohol.

c. **EMBEDDING AND CUTTING.** Blocks after fixation are treated as follows, the first five steps being carried out in wide-mouthed, tightly corked or screw cap bottles:

- (1) Place in 80 percent alcohol 8 hours or more.
- (2) Place in 95 percent alcohol 6 to 8 hours.
- (3) Place in absolute alcohol overnight.
- (4) Place in chloroform 2 to 4 hours.
- (5) Place in chloroform saturated with paraffin 2 to 4 hours at 37° C.
- (6) Place in paraffin, two changes each 2 to 4 hours, in oven.
- (7) Embed in paper or metal forms, with desired surface down, being sure to eliminate air bubbles. To prevent crystallization of paraffin, the mold should be immersed in ice water while the paraffin is still melted.
- (8) Trim block so that opposite edges about tissue are parallel, leaving narrow margin of paraffin.
- (9) Mount on metal block holder by heating latter, pressing on block and immersing all in ice water.
- (10) Cut sections at 5 or 6 microns. Be sure knife is sharp and tightly clamped in the microtome, that its edge

inclines toward the block just enough so that the block misses the back surface of the knife, and that the lower edges of the block and the knife edge are parallel.

- (11) Lay sections on surface of water sufficiently warm to insure complete spreading of section (40° to 50° C.). If the sections are in ribbons, they may be separated by touching while in the water with the edge of a heated scalpel.
- (12) Float the section onto a slide that has been very lightly smeared with Mayer's albumin and wiped off on the palm of the hand.
- (13) Drain off water and place slide in oven for $\frac{1}{2}$ hour to fix albumin.

d. STAINING. In the following steps, use a series of Coplin jars lined up in proper order:

- (1) Remove paraffin by immersing slide in xylene for several minutes, then in absolute alcohol 1 minute.
- (2) Place in 95 percent alcohol 1 minute.
- (3) Place in Lugol's iodine-potassium iodide solution until sections are brown.
- (4) Wash quickly in tap water.
- (5) Bleach in 5 percent sodium thiosulfate solution.

Note. Procedures 3, 4, and 5 are necessary only after fixatives containing mercuric chloride.

- (6) Wash in tap water.
- (7) Stain in Harris' hematoxylin, freshly filtered, 5 minutes, or longer if necessary.
- (8) Rinse in water.
- (9) Differentiate in acid alcohol (1 ml hydrochloric acid in 99 ml of 70 percent alcohol) a few seconds.
- (10) Wash in water 30 seconds.
- (11) Blue in ammonia alcohol (5 drops of ammonia water in 50 ml of 80 percent alcohol) 30 seconds.
- (12) Wash in water 2 minutes.
- (13) Stain in eosin 2 minutes.
- (14) Rinse in 95 percent alcohol to remove excess eosin.
- (15) Absolute alcohol 1 minute.
- (16) Rinse in xylene, two changes, 2 minutes each.
- (17) Mount in Canada balsam or clarite.

520. Rapid Paraffin Method (Mallory and Wright, method No. 3)

a. Tissues already fixed in formalin or fresh tissues boiled 2 to 3 minutes in 10 percent formalin may be used. Blocks should not be more than 5 millimeters thick.

Acetone, four changes, 20 to 30 minutes each.

Benzene, two changes, 30 minutes each.

Paraffin, two changes, 45 minutes each (or one change 15 minutes in vacuo).

b. Following the above method, proceed as in step (6) under routine paraffin method. (See par. 519c.)

Section III. SPECIAL STAINS

521. Stain for Fat

a. Rinse frozen sections briefly in 70 percent alcohol.

b. Stain for 3 minutes in oil red O solution. Use a closed container to prevent precipitate from forming on the section.

Oil Red O Solution:

Oil Red O	1 gm
Acetone	50 ml
Alcohol (70 per cent)	50 ml

If oil red O is not available, Sudan III or Sudan IV may be substituted.

c. Rinse in 70 percent alcohol.

d. Wash in water.

e. Counterstain in Harris' hematoxylin for 1 to 2 minutes.

f. Rinse in tap water until section is blue.

g. Mount in glycerine jelly or glycerol.

Glycerine Jelly:

Gelatin	10 gm
Distilled water	60 ml
Heat to dissolve the gelatin.	
Glycerine	70 ml
Phenol	1 ml

h. Results: Fat is stained red.

522. Kinyoun's Stain for Acid Fast Bacilli (formalin fixation is preferred and the staining should be checked by the use of known controls)

a. Remove paraffin in xylene (two changes), absolute alcohol for 1 minute, 95 percent alcohol for 1 minute, and water for 1 minute.

b. Stain with Kinyoun's carbol-fuchsin for 1 hour at room temperature.

Kinyoun's Carbol-Fuchsin Solution:

Basic fuchsin (rosaniline hydrochloride)	4 gm
Phenol	8 gm
Alcohol (95 percent)	20 ml
Distilled water	100 ml

c. Rinse in water.

d. Decolorize in two changes of 1 percent hydrochloric acid

in 95 percent alcohol until only the thicker portions are pink (1 to 2 minutes).

e. Wash in water until a pale pink.

f. Counterstain with 0.2 to 1.0 percent methylene blue in 0.5 percent acetic acid solution.

g. Wash quickly.

h. Place in 95 percent alcohol, absolute alcohol, and xylene (two changes).

i. Mount in balsam or clarite.

j. Results: Acid fast bacteria stain red.

523. Brown and Brenn Stain for Gram Positive and Gram Negative Bacteria in Tissues

a. Deparaffinize to distilled water.

b. In a small vial, mix 3 drops of 5 percent aqueous solution of chemically pure anhydrous sodium bicarbonate containing 0.25 percent phenol as a preservative, with about 2 ml of 1 percent aqueous solution of crystal violet. Immediately pour the mixture on the slide and stain for a few minutes.

c. Wash quickly in water.

d. Cover with Gram's solution for 1 minute.

Gram's Solution:

Iodine	1 gm
Potassium iodide	2 gm
Distilled water	300 ml

Store in a brown bottle.

e. Wash with water and blot.

f. Decolorize in acetone, dropping it on the slide until color fails to appear.

g. Wash in water.

h. Stain in 0.3 percent aqueous basic fuchsin for 5 minutes.

i. Wash in water; blot but do not allow section to dry.

j. Pass through acetone.

k. Decolorize and differentiate by dipping into a solution of 0.1 gm picric acid in 100 ml of acetone until the section becomes a yellowish pink.

l. Pass successively through acetone, equal parts of acetone and xylene, and xylene.

m. Mount in balsam or clarite.

n. Results: Gram positive bacteria stain blue. Gram negative bacteria stain red. Controls of known organisms should be used.

524. Wilder's Stain for Reticulum

a. Deparaffinize to distilled water.

b. Place in 10 percent phosphomolybdic acid for 1 minute.

- c. Rinse in distilled water.
- d. Dip in 1 percent uranium nitrate (sodium free) solution—5 seconds or less.
- e. Wash 10 to 20 seconds in distilled water.
- f. Place in silver diamino hydroxide for 1 minute.
(Preparation: to 5 ml of 10.2 percent silver nitrate solution add ammonium hydroxide, drop by drop, until the precipitate which forms is dissolved. Add 5 ml of 3.1 percent sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonium hydroxide; make up to 50 ml with distilled water.)
- g. Dip quickly in 95 percent alcohol and place at once in the reducing solution.
- h. Reduce for 1 minute in the following solution:

Uranium nitrate 1 percent aqueous solution	1.5 ml
Neutral formalin (40 percent) (neutralized with magnesium carbonate)	5.0 ml
Distilled water	50.0 ml
- i. Wash in distilled water.
- j. Place in 0.2 percent gold chloride (Merck's reagent) solution for 1 minute.
- k. Rinse in distilled water.
- l. Fix in 5 percent sodium thiosulfate solution for 1 to 2 minutes.
- m. Wash well in tap water.
- n. Dehydrate in 95 percent alcohol and absolute alcohol and clear in xylene.
- o. Mount in clarite or balsam.
- p. Results: Reticulum stains black.

525. Masson's (Modified) Trichrome Stain

- a. Deparaffinize sections to distilled water.
- b. If sections are not Bouin's fixed, place in Bouin's solution at room temperature overnight or in the incubator for 1 hour.
- c. Wash well in running water.
- d. Stain in Weigert's iron chloride hematoxylin for 3 minutes. (par. 517g.)
- e. Rinse in water.
- f. Stain in Biebrich scarlet solution for 10 minutes.
Biebrich Scarlet Solution:

Glacial acetic acid	1 ml
Acid fuchsin 1 percent	10 ml
Biebrich scarlet 1 percent	90 ml
- g. Rinse in distilled water.
- h. Mordant for 3 minutes in phosphomolybdic-phosphotungstic acid mixture. (Equal parts of a 5 percent solution of each acid.)

i. Stain for 5 minutes in aniline blue stain.

Aniline blue stain:

Boil 100 ml of distilled water. When boiling, remove from flame and add 2 gm of aniline blue. Boil again to dissolve the dye. Add 2.5 ml glacial acetic acid. Cool and filter.

j. Differentiate for 1 minute in 1 percent aqueous acetic acid.

k. Rinse in water.

l. Dehydrate and clear.

m. Mount in balsam or clarite.

n. To prevent fading, the balsam must be made acid by adding a few crystals of salicylic acid to 30 ml of balsam.

526. Best's Carmine Stain for Glycogen

a. Fix tissues in either absolute alcohol or Carnoy's solution. (Glacial acetic acid 10 ml, chloroform 30 ml, absolute alcohol 60 ml.)

b. Embed in paraffin.

c. Deparaffinize to absolute alcohol.

d. Soak 5 minutes or more in 1.5 percent celloidin (Parlodion) in equal parts absolute alcohol and ether, drain, wipe back of slide, harden in chloroform, 5 to 10 minutes, and rinse successively in 95 percent alcohol, 80 percent alcohol and water.

e. Stain sections deeply with Harris' hematoxylin 2 minutes.

f. Wash in 80 percent alcohol, two changes.

g. Decolorize with acid alcohol, if necessary.

h. Wash in 80 percent alcohol, two changes.

i. Stain for 30 minutes to 1 hour in a covered dish in the following freshly prepared stain:

Stock carmine solution	20 ml
Ammonium hydroxide (28 percent NH_3)	30 ml
Methyl alcohol	30 ml

Stock Carmine Solution:

Carmine	2 gm
Potassium carbonate	1 gm
Potassium chloride	5 gm
Distilled water	60 ml

Boil gently and cautiously for several minutes until the color darkens. After cooling, add 20 ml of concentrated ammonium hydroxide (28 percent NH_3). Ripen for 24 hours and store in the refrigerator. This stock solution may be kept for several weeks only.

j. Differentiate in the following mixture:

Absolute alcohol	20 ml
Methyl alcohol	40 ml
Distilled water	100 ml

k. Dehydrate in acetone and acetone-xylene. Clear in xylene.

l. Mount in clarite or balsam.

m. Results: Glycogen stains pink to red.

527. Lieb's Permanent Stain for Amyloid

- a. Deparaffinize to distilled water.
- b. Stain 1 to 2 minutes in crystal violet solution.

Crystal Violet Solution:

Hydrochloric acid	1 ml
Crystal violet, saturated alcoholic solution	10 ml
Distilled water	300 ml

- c. Rinse in water.
- d. Mount in abopon (50 gm to 25 ml water). The color will fade with balsam or clarite.
- e. Results: Amyloid stains red.

528. Von Kossa's Silver Nitrate Method (for Staining Calcium Phosphate)

- a. Fix tissues in 80 to 95 percent alcohol or neutral formalin.
- b. Cut frozen sections of nondecalcified or not completely decalcified material, or embed in paraffin.
- c. Rinse sections in distilled water.
- d. Place in a 5 percent aqueous solution of silver nitrate 10 to 60 minutes and expose to a strong light.
- e. Wash thoroughly in distilled water.
- f. Reduce for a few minutes in a 5 percent solution of sodium thiosulfate to remove excess of silver nitrate.
- g. Wash thoroughly in distilled water.
- h. Counterstain in 1 percent aqueous safranin O.
- i. Dehydrate in 95 percent and absolute alcohol.
- j. Clear in xylene and mount in balsam, or clarite.

Note. The calcium as phosphate is stained a deep black where it occurs in masses; finely dispersed granules do not stain deeply. The nuclei are red.

529. Perle (Modified) Iron Reaction

- a. Deparaffinize to distilled water.
- b. Place sections for 30 minutes in equal parts of 5 percent aqueous potassium ferrocyanide and 5 percent hydrochloric acid.
- c. Wash in distilled water.
- d. Lightly counterstain in eosin.
- e. Dehydrate in 95 percent alcohol and absolute alcohol. Clear in xylene.
- f. Mount in balsam or clarite.
- g. Results: Iron is stained blue.

530. Mayer's Mucicarmine Stain for Mucin

- a. Deparaffinize to distilled water.
- b. Stain section for 30 minutes or longer in mucicarmine stain diluted 1 to 10 with distilled water or 60 percent alcohol.

Mucicarmine Stain:

Carmin 1.0 gm
Aluminum chloride, anhydrous 0.5 gm
Distilled water 2.0 ml
Combine and heat over a small flame, stirring constantly, until the reddish mixture becomes a dark color. Gradually add while stirring, 100 ml of 50 percent alcohol. Let the solution stand for 24 hours and filter. This stock solution keeps well.

- c. Wash quickly in water.
- d. Dehydrate quickly through 95 percent alcohol and absolute alcohol.
- e. Clear in xylene and mount in clarite or balsam.
- f. Results: Mucin stains red.

531. Mallory's Phosphotungstic Acid Hematoxylin (stain for glia and fibrils; preferably fixed in Zenker's fluid; if fixed in formalin, mordant sections in saturated mercuric chloride at 56° C. for 3 hours)

- a. Carry section through to water.
- b. Lugol's iodine-potassium iodide solution until sections are brown.

- c. Wash quickly in tap water.
- d. Bleach in 5 percent sodium thiosulfate.
- e. Wash in tap water.
- f. Place in 0.25 percent aqueous potassium permanganate solution for 5 minutes.

- g. Wash in distilled water.
- h. Bleach in 5 percent oxalic acid for 5 minutes.
- i. Wash in distilled water.

- j. Stain in the following solution for 3 hours:

Hematoxylin 0.1 gm
Phosphotungstic acid 2 gm
Distilled water 100 ml

Note. For ripening, add 10 ml of 0.25-percent aqueous potassium permanganate solution, or age for several months.

- k. Rinse in water.
- l. Differentiate and dehydrate in 95 percent alcohol and absolute alcohol.
- m. Clear in xylene (two changes).
- n. Mount in balsam, or clarite.

Note. The nuclei, centrosomes, fibroglia, myoglia, fibrin, and contractile elements of striated muscle are stained blue, the collagen, reddish brown and the cartilage and bone, various shades of red.

532. Weigert-Van Gieson Stain (for connective and elastic tissue)

- a. Carry sections through to water.
- b. Stain in following solution 15 minutes at 56° C. (paraffin oven), heating the stain slightly before using:

Basic fuchsin	2 gm
Resorcinol (fresh and crystalline)	4 gm
Distilled water	200 ml

Mix in porcelain dish and heat. When boiling briskly, add 25 ml of 29-percent aqueous solution of ferric chloride. Boil until a precipitate forms (2 to 5 minutes). Cool and filter. Return filter paper and precipitate to porcelain dish, which will contain a residue of precipitate and let dry thoroughly in an oven at 37° C. Add 200 ml of 95 percent alcohol and boil *carefully* over a low flame, stirring constantly until the precipitate is completely dissolved. Cool and filter; filter paper should contain no granules or metallic residue. Add 4 ml of concentrated hydrochloric acid.

- c. Wash thoroughly in 95 percent alcohol.
- d. Wash in 2 percent alcohol.
- e. Wash in water.
- f. Stain with Weigert's iron hematoxylin. (See par. 517g.)
- g. Wash in water.
- h. Stain in following solution 3 minutes:

Acid fuchsin	0.1 gm
Saturated aqueous picric acid	100 ml
- i. Rinse in 95 percent alcohol.
- j. Run through alcohol and xylene.
- k. Mount in balsam, or preferably clarite.
- l. Results: Elastic tissue stains black.

533. Levaditi's Stain (for *Treponema pallidum*)

- a. Fix tissue (blocks not over 1 mm thick) in 10 percent formalin.
- b. Rinse in water, and place in 95 percent alcohol for 24 hours.
- c. Place in distilled water until the tissue sinks to the bottom of the container.
- d. Place in 1.5 percent aqueous silver nitrate solution and keep in incubator at 38° C. for 4 days; a stronger solution of silver nitrate is better for tissues removed at biopsy.
- e. The following step must be done in the dark. Wash in distilled water and place in the following solution for 48 hours at room temperature:

Pyrogallic acid	3 gm
Formalin (40 percent)	5 ml
Distilled water	100 ml

- f. Wash in distilled water.
- g. Place in 80 percent alcohol for 2 hours.
- h. Place in 95 percent alcohol for 5 to 10 hours.
- i. Place in absolute alcohol overnight.
- j. Run through chloroform and paraffin as usual; embed.
- k. Cut sections, and remove paraffin in two or three changes of pure xylene.
- l. Mount in balsam, or clarite.

Note. The organisms are stained intensely black by the precipitation of metallic silver upon them; the reticulum stains brown, whereas the other elements of the tissue are yellow.

534. Warthin Starry Method for *Treponema Pallidum*

- a. Solutions.
 - (1) One percent aqueous citric acid.
 - (2) To 5 liters of doubly distilled water, add enough 1 percent citric acid to establish a pH of 4.4. The pH can be determined satisfactorily by using bromcresol green as an indicator.
 - (3) Using 200 ml of acidulated water, make a 2 percent solution of silver nitrate.
 - (4) Using 33 ml of acidulated water, make a 3 percent solution of hydroquinone. Prepare fresh before use.
 - (5) Using 200 ml of acidulated water, make a 5 percent solution of gelatin, heating in the oven at 55° to 60° C. for 1 hour. Two ml of 1:10,000 merthiolate may be added as a preservative.
- b. Deparaffinize to distilled water.
- c. Rinse in acidulated water.
- d. Place in 1 percent silver nitrate solution in acidulated water in oven at 55° to 60° C. for 30 minutes.
- e. Place slide, section side up, on glass rods in a Petri dish and pour developer over it. The developer is prepared just before use by mixing solutions (3), (4), and (5) as described above, in the following proportions:

2 percent silver nitrate (warm in oven at 55° to 60° C.)	3 ml
5 percent gelatin (warm in oven at 55° to 60° C.)	15 ml
3 percent hydroquinone, freshly prepared	1 ml
- f. Allow the sections to develop in this mixture until they turn a light golden brown or yellow, depending upon the kind of tissue.
- g. Check development quickly by rinsing in warm tap water. (55° to 60° C.)
- h. Rinse in distilled water at room temperature.
- i. Dehydrate and clear in alcohols and xylene.
- j. Mount in clarite or balsam.
- k. Results: Spirochetes are black on a yellow background.

535. Giemsa's Stain

a. REAGENTS.

- (1) *Stock acetone.* To 500 ml of acetone, add 0.1 ml of glacial acetic acid.
- (2) *Stock Giemsa's stain.* Place 0.6 gm of Giemsa's stain (certified powder) in 50 ml of glycerol at 55° to 60° C. for 1½ to 2 hours; to this add 50 ml of methyl alcohol (methanol) that has been heated to the same temperature.

Caution: Heat alcohol in a screwtop bottle in a paraffin oven or in hot water; keep away from flame. When the solution has cooled, filter and keep in a tightly stoppered bottle. (See par. 464.)

b. TECHNIC.

- (1) Bring sections (Zenker-, Regaud-, Bouin-, or formalin-fixed) to water in the usual way for each technic.
- (2) Rinse in distilled water.
- (3) Place in dilute Giemsa's stain 3 to 4 hours observing intensity of stain at the end of each hour (should be somewhat overstained); to obtain the optimal staining after various fixations, the pH of the stain should be adjusted by the use of M/15 citric acid and disodium phosphate buffers (dissolved in 25 percent methyl alcohol); the following amounts should be added to 40 ml of dilute (1:20) Giemsa's stain:

Fixative	Citric acid	Disodium phosphate
Formalin.....	1.2 ml.....	0.8 ml
Zenker.....	1.0 ml.....	1.0 ml
Bouin.....	0.8 ml.....	1.2 ml
Regaud.....	1.1 ml.....	0.9 ml

Note. The addition of 5 ml of acetone to the staining solution accelerates staining.

- (4) Remove from stain, and wave in air to dry slightly.
- (5) Dehydrate in the stock acetone under observation if no buffer was used, otherwise dehydrate quickly in pure acetone.
- (6) Drain slightly and place in xylene (two changes); in moist climates use mixture of equal parts of xylene and acetone before the xylene.
- (7) Mount in cedarwood oil (immersion), or preferably clarite.

536. Wolbach's Giemsa Stain

a. Giemsa Stain (Stock Solution) :

Giemsa stain, certified powder 0.50 gm
Glycerol 33.00 ml

Place in oven at 55° to 60° C. for 2 hours and add 50 ml of methyl alcohol which has been heated in a screw-top bottle in an oven or water bath to 55° to 60° C. Keep away from flame. When the solution has cooled, filter and keep in a tightly stoppered bottle.

b. Wolbach's Staining Solution :

Giemsa stock solution 1.25 ml
Methyl alcohol 1.50 ml
Distilled water 50.00 ml

c. Deparaffinize to distilled water.

d. Place in Wolbach's Giemsa for 24 hours.

e. Differentiate in white rosin alcohol until sections are purplish-pink. Do not decolorize too much.

f. Rosin-alcohol.

Saturated solution of white rosin in alcohol 5 ml
Alcohol, 95 per cent 45 ml

g. Absolute alcohol, two rapid changes.

h. Xylene, two changes.

i. Mount in clarite or cedarwood oil (immersion). The stain will fade in balsam.

537. Decalcification

a. After fixation in 10 percent formalin, decalcify small pieces of bone by either of the following methods:

(1) *Nitric acid method.*

- (a) Place specimen in 5 percent nitric acid in 0.85 percent saline solution.
- (b) Transfer specimen to fresh solution of acid several times during decalcification. Temporal bones and teeth should be transferred every 24 hours. Decalcification may be considered complete when bones bend easily. Several days will be required for decalcification depending upon the specimen.
- (c) Place in 10 percent formalin containing magnesium carbonate, to neutralize the acid.
- (d) Wash in running water for 24 to 48 hours.

(2) *Formic acid method.*

- (a) Place the specimen in a solution made by combining 1 and 2. This solution keeps well.
 1. Sodium citrate 50 gm
Distilled water 250 ml
 2. 90 percent formic acid 125 ml
Distilled water 125 ml

- (b) Decalcify 5 to 14 days, changing the solution daily.
- (c) Wash in running water 2 to 8 hours.
- (d) This method gives a better staining reaction than the nitric acid method.

b. After decalcification, tissues are dehydrated and embedded in paraffin.

c. Stain sections with hematoxylin and eosin for 15 minutes using Harris' hematoxylin without acetic acid; rinse in tap water; differentiate in 1 percent acid alcohol; rinse in tap water; blue sections in 1 percent aqueous lithium carbonate; wash in running tap water for 5 minutes. Counterstain in alcoholic eosin for 1 minute; dehydrate, clear, and mount in balsam.

538. Periodic Acid-Schiff Reaction for Glycogen, Mucin and Reticulum

a. Fixation in 10 percent formalin in alcohol is preferable but 10 percent aqueous formalin may be used.

b. Deparaffinize to water.

c. Place in 0.25 percent periodic acid for 5 minutes.

d. Rinse in distilled water.

e. Place in Schiff's reagent for 15 minutes.

Schiff's Reagent:

Dissolve 1 gm basic fuchsin in 200 ml hot (90° to 95° C.) distilled water. Allow to cool to 50° to 60° C. and filter. Cool to 25° C. and add 1 gm sodium bisulfite and 20 ml normal hydrochloric acid (see *m.* below). Stopper tightly and place in the dark at room temperature until the solution darkens to a straw or amber color. This may require 24 to 48 hours. Store in the dark at 5° C. At this temperature, the solution remains active for some months. It should be discarded if a pink tint appears.

f. Rinse 2 minutes in each of three changes of a sulfite bath containing 6 ml 10 percent sodium bisulfite, 5 ml normal hydrochloric acid and 100 ml distilled water.

g. Wash in running water 3 to 5 minutes.

h. Counterstain very lightly in Harris' hematoxylin.

i. Wash in water.

j. Dehydrate and clear.

k. Mount in balsam or clarite.

l. Results: Glycogen dark purple, mucin reddish-purple to violet, reticulum reddish-purple.

m. Normal hydrochloric acid may be made with sufficient accuracy by diluting 83.5 ml concentrated hydrochloric acid 36.7 percent, specific gravity 1.19 to 1000 ml in a volumetric flask.

539. Bauer-Feulgen Technic for Fungi

- a. Fixation in 10 percent formalin in alcohol is preferable but fixation in 10 percent aqueous formalin may be used.
- b. Deparaffinize to water.
- c. Mordant 1 hour in 4 percent chromic acid.
- d. Wash in tap water 10 minutes.
- e. Place for 15 minutes in Bauer's modification of Schiff's reagent (par. 538e) which uses 1 gm basic fuchsin, 1 gm sodium bisulfite and 20 ml normal hydrochloric acid in 100 ml water rather than in 200 ml water.
- f. Rinse for 1 to 2 minutes in each of three changes of twentieth molar sodium bisulfite. M/20 sodium bisulfite may be made from a stock molar solution (10.4 percent), by diluting 6 ml of the stock solution with 114 ml of tap water.
- g. Wash in running water 10 minutes until the section begins to turn pink.
- h. Counterstain lightly in hematoxylin.
- i. Dehydrate and clear.
- j. Mount in balsam or permount.
- k. Results: Fungi stain red.

540. Negri Bodies in Paraffin Section (Schleifstein)¹

- a. Use Zenker-fixed material.
- b. Remove paraffin in xylene and rinse in absolute alcohol 1 minute and in 95 percent alcohol 1 minute.
- c. Rinse in Lugol's solution until sections are brown and wash quickly in tap water.
- d. Bleach in 5 percent sodium thiosulfate solution.
- e. Wash in tap water.
- f. Flood with freshly prepared stain (working solution) and steam gently for 5 minutes.
- g. Let cool and wash quickly in tap water.
- h. Differentiate carefully in 80 percent ethyl alcohol until faint violet.
- i. Dehydrate rapidly in 95 percent alcohol and absolute alcohol.
- j. Clear in xylene and mount in clarite.
- k. Stock solution:

Basic fuchsin	1.8 gm
Methylene blue	1.0 gm
Glycerol	100.0 ml
Methyl alcohol	100.0 ml

Shake well before use.

l. Working Solution:

Stock solution 1 drop
1:40,000 potassium hydroxide (or tap water,
if it is alkaline) 2 ml

m. Results: Negri bodies stain red.

541. Myelin Sheath Stain—Paraffin Section (Weil)

a. Cut sections at 15 u.

b. Deparaffinize to water.

c. Mordant overnight in 4 percent ferric ammonium sulfate.

d. Stain at 50° to 55° C. in equal parts of A & B for 10 to 15 minutes. Mix from stock bottles just before use, adding solution A to solution B.

A. Ferric ammonium sulfate, 4 percent.

B. Absolute alcohol solution of hematoxylin,
1 percent (1 to 5 days old, only) 10 ml
Distilled water 90 ml

e. Wash twice in tap water.

f. Differentiate in 1 percent ferric ammonium sulfate. Control with the microscope.

g. Wash three times in tap water.

Complete differentiation in:

Borax (sodium borate) 10.0 gm
Potassium ferricyanide 12.5 gm
Distilled water 1,000.0 ml

h. Wash twice in tap water.

i. Wash 30 seconds in dilute ammonia water (6 drops, 28 percent ammonia in 100 ml water).

j. Wash in distilled water.

k. Dehydrate, clear and mount.

APPENDIX

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